BRNO UNIVERSITY OF TECHNOLOGY
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EFFECT OF CROWDING AGENTS ON DNA-SURFACTANT INTERACTION
VLIV "ZAPLŇOVACÍCH" ČINIDEL NA INTERAKCE DNA-TENZID

MASTER'S THESIS
DIPLOMOVÁ PRÁCE

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VEDOUCÍ PRÁCE

BRNO 2017

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
FAKULTA CHEMICKÁ

Zadání diplomové práce

Číslo práce:	FCH-DIP0944/2015
Ústav:	Ústav fyzikální a spotřební chemie
Studentka:	**Bc. Šárka Sovová**
Studijní program:	Chemie pro medicínské aplikace
Studijní obor:	Chemie pro medicínské aplikace
Vedoucí práce:	**prof. Ing. Miloslav Pekař, CSc.**
Akademický rok:	2016/17

Název diplomové práce:

Vliv "zaplňovacích" činidel na interakce DNA-tenzid

Zadání diplomové práce:

1) Literature review of DNA-surfactant interaction with emphasis on cationic surfactants.
2) Preparation of DNA by PCR method.
3) Experimental measurements. The experimental measurements will include: (i) the determination of surfactant (CTAB) critical micellar concentration in the crowding conditions by tensiometry; (ii) the evaluation of the effect of crowding agents on the interaction between DNA and CTAB using dye exclusion assays, and electrophoretic mobility; and (iii) the impact of CTAB, in a crowded environment, on DNA protection against degradation and digestion, using DNA fingerprinting assays.
4) Data evaluation and discussion of results.
5) Writing of the thesis.

Termín odevzdání diplomové práce: 5.5.2017

Diplomová práce se odevzdává v děkanem stanoveném počtu exemplářů na sekretariát ústavu. Toto zadání je součástí diplomové práce.

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V Brně dne 31.1.2017

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ABSTRAKT

Tato diplomová práce se zabývá vlivem zaplňovacích činidel na interakce v systému DNA-tenzid. DNA o velikosti 4017 párů bází byla připravena polymerázovou řetězovou reakcí, jako templát byl použit plasmid pSB-E1g. Polyetylen glykol (PEG) byl použit jako zaplňovací činidlo a jeho vliv na DNA-tenzid interakce byl zkoumán experimenty založenými na fluorescenci a gelové elektroforéze. Také byl studován vliv iontové síly za použití NaBr na interakce DNA-tenzid za použití zaplňovacího činidla. Data byla vyhodnocena a evaluována v této práci. V úvahu byl brán i možný vliv polyetylen glykolu na kritickou micelarní koncentraci (CMC) tenzidu, bylo provedeno měření CMC pomocí ultrazvuku s vysokým rozlišením, avšak nebyl zjištěn žádný značný vliv zaplňovacího činidla na CMC tenzidu. Část této práce bude zahrnuta v publikaci s anglickým názvem Combined role of macromolecular crowding and cationic surfactant in efficient DNA condensation.

ABSTRACT

The diploma thesis focuses on DNA-surfactant interaction in crowding environment. DNA of 4017 base pair size was prepared using polymerase chain reaction and template plasmid pSB-E1g. The crowding environment was mimicked by polyethylene glycol and its effect on the interactions of DNA-surfactant system was investigated by dye exclusion study, electrophoretic mobility shift assay and DNase protection assay. Also the effect of ionic strength on DNA-surfactant interaction in crowding environment, using NaBr, was investigated. Data were evaluated and discussed in this work. The effect of crowding agent on surfactant CMC was studied using high resolution ultrasonic velocity. It was found that there is no significant effect of PEG on surfactant CMC. This work is going to be part of the scientific publication with title Combined role of macromolecular crowding and cationic surfactant in efficient DNA condensation.

KLÍČOVÁ SLOVA

DNA, tenzid, zaplňování, polymerázová řetězová reakce, fluorescence, gelová elektroforéza, ultrazvuk s vysokým rozlišením

KEYWORDS

DNA, surfactant, crowding, polymer chain reaction, fluorescence study, gel electrophoresis, high resolution ultrasonic spectroscopy

SOVOVÁ, Š. *Vliv "zaplňovacích" činidel na interakce DNA-tenzid.* Brno: Vysoké učení technické v Brně, Fakulta chemická, 2017. 61 s. Vedoucí diplomové práce prof. Ing. Miloslav Pekař, CSc..

DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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student's signature

ACKNOWLEDGEMENT

Firstly, I would like to thank associate professor Rita de Sousa Dias and PhD student Sravani Ramisetti from Norwegian University of Science and Technology in Trondheim, Norway, where I worked on my thesis, for their willingness to help me with solving many obstacles during my research. Then I would like to Norway grants and project NF-CZ07-INS-5-192-2015 for one year financial support during my stay at NTNU and also Ministry of Education for financial support. My thanks belong also to Mgr. Alena Sýkorová for paperwork during my application for studying abroad. Last but not least, I would like to thank to Professor Miloslav Pekař for support and approval of my stay abroad. Also, I would like to thank to Dr. Andrea Kargerová and Dr. Jitka Krouská for help with measurements performed at Materials research centre. And at the end, I would like to thank to my both international and Czech friends for support.

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1 Introduction

Molecular crowding is like gravity – always present and living organisms have to deal with it [1]. Most researchers do not take macromolecular crowding into consideration. However, ongoing processes in living organisms take place in highly concentrated medium of macromolecules (50-400 $g \cdot L^{-1}$) [2]. Rather than concentrated, the medium is referred to be crowded, because single macromolecules are not often present in high concentration; however the overall amount of macromolecules occupies a significant fraction of the volume of the medium [3]. In some media, for example blood plasma, the concentration of macromolecules reaches the incredible amount of 80 $g \cdot L^{-1}$ [2, 4]. For example in pharmaceutical research, crowding environment should be considered. The binding of macromolecular ligand to its complementary site can work excellently under laboratory conditions, however in the living system binding may not take place at all. The binding constant is influenced by the volume occupancy of the surrounding medium and might be therefore changed by one or more orders of magnitude [3]. On the other hand, the molecular crowding effects on properties of the biomolecules are unclear [5].

Since polyethylene glycol (PEG) is soluble, nontoxic and biocompatible neutral polymer, it is widely used in different industries, medicine and pharmaceutical field [6]. On the other hand, we should be aware of phase separation between DNA and neutral cosolutes, for example PEG [4].

DNA–surfactant systems are widely studied. It has been found that hydrophilic part of the surfactant molecules, if positively charged, bind to DNA chain through Coulomb attractive interactions and complexes are stabilized by hydrophobic interaction of hydrophobic part [7]. In the system with low cationic surfactant concentration, free cations are present and DNA is anionic in aqueous solution. This allows cationic surfactant molecules to get closer to DNA and interact through electrostatic attraction [8]. Using fluorescence microscopy; three distinct DNA conformation states were found in solution. Depending on the CTAB concentration, the conformation changed from elongated coil state through the coexistence between coil and globule to the final globule state [7].

Guos research team studied the interaction between DNA and three cationic surfactant with different alkyl chain lengths using UV–vis spectroscopy, fluorescence spectroscopy and viscosity techniques. They confirmed that all three surfactants can interact with DNA. They also estimated their binding modes and compared their interaction strength. They found out that the CTAB molecules with the longest chain gives the strongest interaction with DNA molecules, meaning that interaction strength increases with the increase of the alkyl chain length. They also studied the effect of NaCl on the interaction and found out that NaCl prevents binding between DNA and fluorescent probe and also weaken the interaction between surfactant and DNA [8]. Rimawi and others studied DNA-CTAB aggregates using atomic force microscopy. They dried DNA-CTAB solutions on mica and silicon, reconstituted the dried aggregates in buffer and imaged them in solution. They used functionalized AFM tips and found consistent, almost hexagonal and regular patterns. They also found out that the surface of the aggregate is hydrophilic [9].

Yoshikawa and others studied DNA compaction induced by negatively charged protein, albumin, which was used as a crowding molecule. They used fluorescence microscopy to study the change in the giant T4 DNA molecule conformation and found out that DNA undergoes compact conformation above critical albumin concentration. Moreover, they studied salt effect on DNA compaction and found the opposite effect than widely explained

psi condensation. In other words, NaCl retards the transition from the elongated state to the compact state [10]. Other study which contradicts salt induced (psi) condensation is mentioned by Krotova and co-authors. They studied the conformational properties of DNA in a salt solution in a crowded environment of strongly negatively charged protein albumin. The result of the study was interesting. They found out that low-molecular-weight salt (NaCl in this experiment) prevents the compaction of DNA which is the total opposite of the well-known phenomenon, polymer- and salt-induced DNA condensation. This effect is mentioned to be DNA decompactization due to complex interplay of electrostatic interaction and translational entropy of the counterions [11].

Hou and others studied DNA behaviour in crowding environment. They found out that at high PEG (M_w=6 000g/mol) concentration, the cleavage of DNA by HindIII enzyme is stopped and DNA nanoparticles are formed. They characterized DNA nanoparticles using fluorescence correlation spectroscopy, dynamic light scattering, fluorescence analytical ultracentrifugation and found out that macromolecular crowding (>25% PEG 6 000) promotes formation of nanoparticles with several hundreds of nanometer. They also found out that the formation of DNA nanoparticles is fast and reversible. It should be noted that DNA nanoparticles formation is only possible when both buffer and crowding molecules are present. Neither of them alone can cause DNA nanoparticle formation. The buffer R they used was composed of 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl and 0.1 mg/mL BSA) [12].
Zinchenko and Yoshikawa studied DNA compaction in crowded environment induced by alkali metal salts, LiCl, NaCl, KCl, RbCl, CsCl. They found out that all of them promote DNA compaction. Fluorescence microscopy was used to investigate T4 DNA interaction with alkali metal salts in the presence of PEG 3 000 or PEG 10 000. They found out that Na^+ produces the greatest DNA compaction. The reason for this is the small size of the hydrated cation which can effectively interact with DNA due to higher Coulomb electrostatic potential and the possible incorporation into DNA minor groove for necessary charge neutralization [13].

Ramisetty and Dias investigated the synergistic role of DNA-binding protein and macromolecular crowding on DNA condensation. Both H-NS and spermine were studied as a DNA-binding agents and PEG was used as a crowding molecule. Exclusion dye assay and Monte Carlo simulations were used. System with low/intermediate spermine concentration and larger PEG concentration shows synergistic effect. Salt effect was also taken into consideration and they found two different regimes. Psi condensation dominates at low spermine concentrations and larger DNA condensation was found in the higher ionic strength. Higher spermine concentration and larger ionic strength led to a more moderate DNA concentration. Therefore, synergism effect was found to be significant at low DNA-binding agent concentration and high ionic strength [14].

The aim of this work is to better understand how crowding agents affect DNA-surfactant interactions. Firstly, polymerase chain reaction and plasmid pSB-E1g from Escherichia Coli are used to produce DNA of the 4 017 base pair size. Investigation was done using dye exclusion assays and electrophoretic mobility. Also the determination of surfactant critical micellar concentration in the crowding conditions was investigated by tensiometry and high resolution ultrasonic spectroscopy. The final goal was to find out the impact of CTAB, in crowded environment, on DNA protection against degradation and digestion using DNA assays.

2 Theoretical part

2.1 Deoxyribonucleic acid

Deoxyribonucleic acid, DNA, is one of the most important biomacromolecules of all known living organisms and viruses. It plays a crucial role in storing the genetic information of organisms. Three-dimensional atomic structure of the DNA was identified by James Watson and Francis Crick in 1950s using X-ray diffraction [15]. DNA is present in our cells as structures, chromosomes, where every chromosome contains up to 100-200 million base pairs [16].

2.1.1 Structure

DNA is a polymer formed by monomeric subunits called nucleotides. Each of them is composed of a five-carbon sugar called deoxyribose, a phosphate group and a nitrogen base which can differ. There are four types of nitrogen base: adenine (A), guanine (G), cytosine (C) and thymine (T) [16, 17].

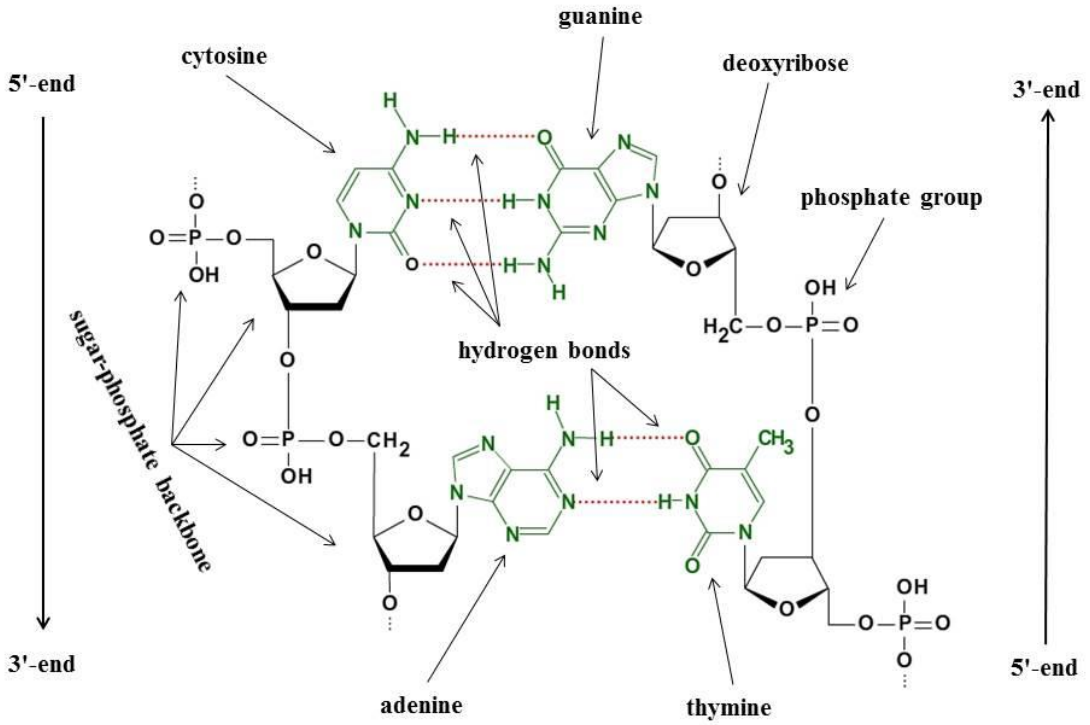

Figure 1: Complementary base pairs in the DNA double helix.

Two individual DNA strands run in opposite directions forming a helical structure [19]. Hydrogen bonds are formed between the bases of nucleotides, holding the two chains together [17]. For this reason, bases are on the inside of the double helix, and sugar-phosphate backbones on the outside (*Figure 1*). The nucleotides are linked together covalently in the chain through the sugars and phosphates. In the other word, it forms a "backbone" of alternating sugars and phosphates [16].

Complementary base pairs are formed according to the base-pair rule and hydrogen bonds are formed between nucleotides. Guanine nucleotides always base pair with cytosine nucleotides and thymine nucleosides with adenine nucleosides [16]. Guanine and cytosine form a base pair with three hydrogen bonds, whereas two hydrogen bonds are formed between adenine and thymine [16].

DNA is strongly negatively charged with one elementary negative charge per each 0.17 nm (projection on the axis) of the double helix with the diameter of 2 nm. On the other hand, DNA can be stretched up to several centimetres depending on the organism [20]. The distance between nucleotides in a chain is 3.4 Å, and thus very large molecules can reach several centimetres, if unfolded.

2.2 Associated colloids

In a very dilute solution, some chemical substances form homogenous solution, also called true solution. With increasing concentration, the molecules aggregates and the solution become colloidal with the particle size 1 – 1000 nm .These aggregated particles are called micelles [21], and the chemical substances able to form micelles are known as associated colloids [5]. During this process, the temperature is important. Micelles are formed only if the temperature is higher than Krafft temperature (T_k), otherwise the solubility of the surfactant is not sufficient for micellisation, and if the concentration is higher than critical micellar concentration (see 2.2.3 below) [21]. These colloids can be reverted by dilution.

2.2.1 Surfactants

The term surfactant refers to the surface active agents, usually organic compounds which lower the interfacial or surface tension [21]. Surfactants are primarily used for removing fats, for example washing clothes and dishes, but are also used in cosmetics and pharmaceutics, due to their ability to self-assembly and formation of structures that can be used to solubilize hydrophobic drugs, for example [18]. Recently, surfactants become more and more investigated in relation with the drug delivery systems [22, 23, 24].

2.2.2 Structure

The ability to solubilize hydrophobic substances is enabled by the dual character of surfactant molecule, meaning that single molecule is composed of two parts – lyophilic "head" and lyophobic "tail", *Figure 2*, [21]. In other words, in aqueous solution, surfactants are amphiphilic molecules with one part that likes water, hydrophilic, and one part disliking water, hydrophobic [5]. The hydrophilic part is polar and gives the molecule the ability to dissolve in water [25]. Hydrophobic part is usually composed of hydrocarbon chain. The dislike for the solvent causes association of lyophobic tail and subsequent micelle formation [25]. Despite their mutual antipathy, these two distinct regions, the head and tail group, cannot leave one another because they are covalently bounded [21].

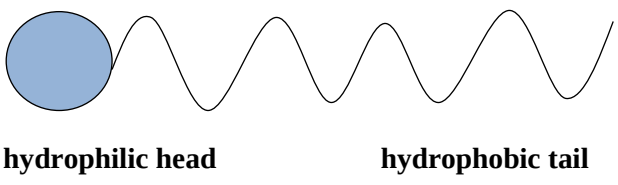

Figure 2: Surfactant molecule.

Surfactants can be divided into three main classes, anionic, cationic and nonionic. The head group can contain charge, in case of anionic surfactant the head group is negatively charged, as for example sulphate, phosphate or carboxylate head group. Cationic surfactants are positively charged and contain, usually, quaternary ammonium or pyridinium head group. The last class, nonionic surfactants are not charged and solubility is ensured by polar groups [21, 25]. Zwitterionic surfactants are surfactants with the net neutral charge; the head group has two oppositely charged species [5].

2.2.3 Aggregation and critical micellar concentration

Amphiphilic structure of the surfactant molecule gives rise to micelle formation at sufficient high surfactant concentrations. In aqueous solution, the polar head groups are oriented outwards of the micelle and nonpolar tail inwards. Micelle formation plays crucial role in dissolving nonpolar molecules in solution. The core of the micelle is hydrophobic and dissolves nonpolar compounds, such as drugs. In nonpolar solvent, reversed micelles are formed (hydrophobic tail heading outwards and hydrophilic inwards) [27]. Aggregation number, average number of surfactant molecules per micelle, is an important characteristic of micelles [28].

The concentration at which aggregation starts is called the critical micellar concentration (CMC) and the arising structures are called micelles. Below the CMC, the solution is composed of single surfactant molecules. At low concentration, surfactants molecules accumulate on the surface in order to minimize the contact of hydrophobic parts with water. Above the CMC, surfactant molecules aggregate into micelles, which means that CMC is the highest concentration at which the surface active agents are present as free molecules. The critical micellar concentration is usually in the range of 10^{-5} and 10^{-3} mol·L^{-1} [27]. In case of ionogenic micelles, polar groups dissociate and charged micelles are surrounded by electric double layer of dissociated counterion [29].

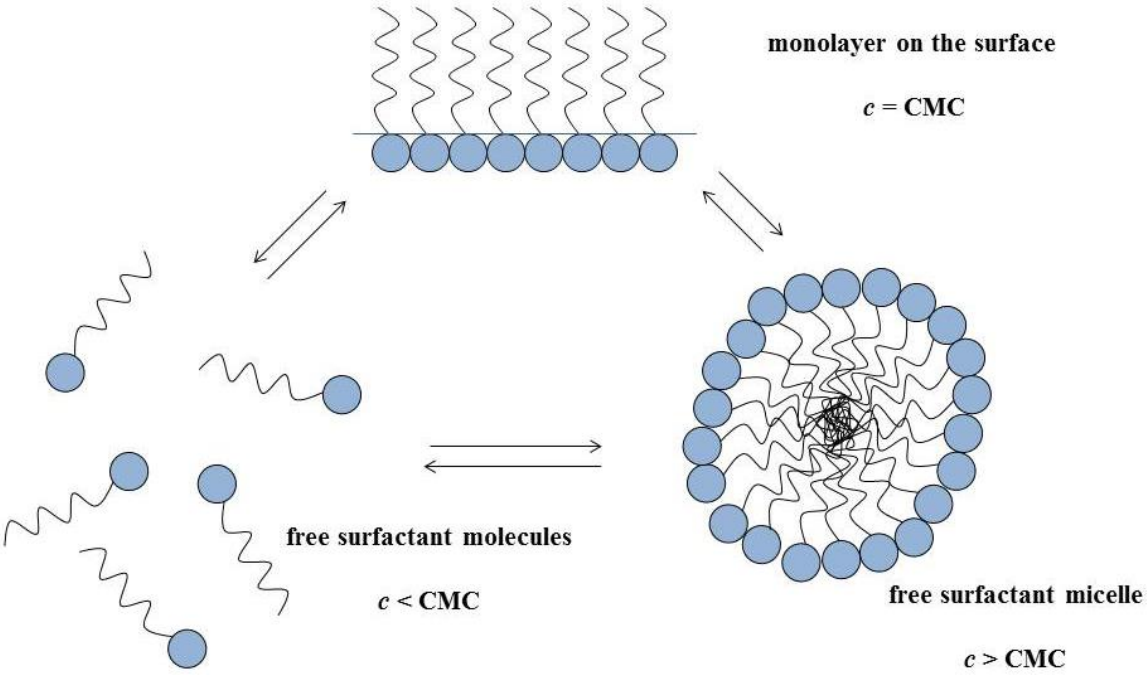

Figure 3: Micellization.

2.3 Molecular crowding

As mentioned in the introduction, biochemical processes take place in very crowded environment. While trying to understand biochemical processes, most of the experiments *in vitro* have been investigated in dilute solution. However, macromolecules present in processes *in vivo* modify ongoing processes (e.g. the rates and the equilibria), so *in vitro* experiments do not reflect those processes accurately [30].

Living cells possess a variety of soluble and insoluble components, such as nucleic acids, proteins and polysaccharides. The single species are not present in high concentrations, but in total, the concentration can reach about 400 $g \cdot L^{-1}$. Molecular crowding involves both macromolecules and small molecules. These crowding agents occupy a significant fraction on the cellular volume, 20–40 % [4].

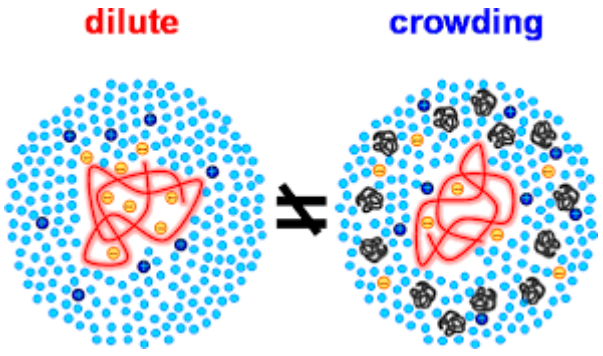

Figure 4: Macromolecular crowding, blue dots represent water [17].

2.3.1 Excluded volume

If one wants to be more accurate, molecular crowding can be termed as the excluded volume effect. Excluded volume is defined as the volume occupied by a molecule which is unavailable for other molecules [19]. The reason is the mutual impenetrability, the main characteristic of all atoms and molecules, and this nonspecific steric repulsion is always present [33, 34]. It is important to take excluded volume into consideration because the dynamic and molecular movement is suppressed by the present molecules of different size [4]. The conformational entropy of the macromolecules in the smaller volume is restricted which means that the free energy on the system increases. That is, excluded volume favours reactions that proceed with volume reduction [35].

2.3.2 Crowding agents

Crowding agents, cosolutes, are used to mimic molecular crowding *in vitro*. One of the criteria that cosolutes should meet is high solubility in water. They should also be inert, since direct interactions between target molecule and the cosolute are unwanted. Either large or small cosolutes can be used. For large ones, different polymer sizes are available. Examples of large cosolutes are PEG, dextran or Ficoll. In the group of commonly used small cosolutes, alcohols, glycols and amino acids can be involved [4].

2.4 Polymerase chain reaction

Polymerase chain reaction, PCR, is an *in vitro* method used to amplify DNA. In other words, it is a simple method capable of making millions of copies of a specific DNA sequence [26]. It was invented in 1980s by Kary Millis, which was awarded the Nobel Prize in Chemistry in 1993 for this. PCR is widely used in research, diagnosis of hereditary or infectious diseases, and the identification of genetic fingerprints [36].

2.4.1 PCR components

The basic reaction mixture is composed of a DNA template, two primers, nucleotides, DNA polymerase and a buffer.

DNA template is usually low-concentrated DNA sample containing the DNA region to be amplified. It can be plasmid DNA, genomic DNA or a small amount of tissue. Short oligonucleotides of DNA with specific sequence are called primers and are the critical part of PCR reaction. Primers of desired sequence can be obtained commercially. Two kinds of primers are basically required for the reaction, 5` end primer and 3` end primer. Complementary base pairing allows 5` end primer to match with the end of the top strand and 3` end primer to other end on the bottom. Thermostable DNA polymerase is an enzyme complex capable of tolerating the high temperatures needed for melting the DNA double strand. During the PCR, DNA polymerase amplifies DNA fragments. Nucleotides, usually known as dNTP mix (ATP, CTP, GTP, TTP), are building blocks from which DNA polymerase makes new DNA molecules. DNA polymerase uses free complementary nucleotides and attaches them to the 3` end of a primer and pairing them with the template DNA. The right pH conditions are maintained by PCR buffer, which also provides necessary ions for enzymes activity [37].

2.4.2 PCR principle

The PCR runs for tens of cycles of three basic steps: denaturation, annealing and elongation. The DNA is copied exponentially, which means 2^{20} amplifications after 20 cycles [38].

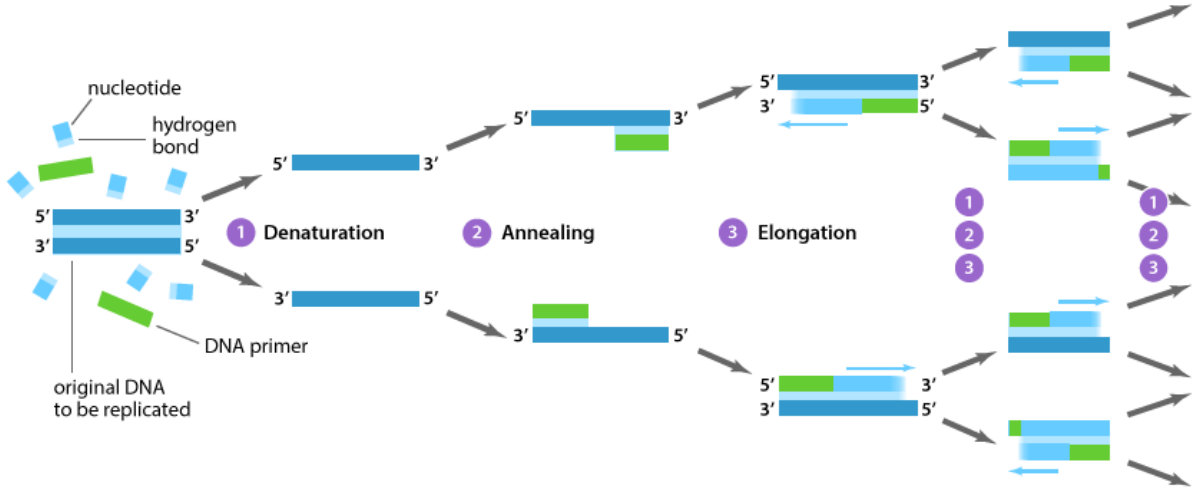

Figure 5: Steps in DNA copying [37].

Firstly, in the denaturation step, the mixture in the thermocycler (PCR machine) is heated to the high temperature of 90 °C or more, which melts or unzips the double-stranded DNA templates (hydrogen bonds are broken). The temperature is decreased in annealing step and DNA primers can anneal to complementary sequences on the templates. As mentioned before, two primers are used, one for each strand of the template [39]. In the elongation step, temperature is increased to reach the optimal DNA polymerase temperature which is around 72 °C [37]. Since DNA polymerase has only 3` end activity, it can only add bases in the direction from 5` end to 3` end, where the primer is annealed [39]. DNA polymerase synthesises a new DNA strand which is complementary to the original one by adding complementary nucleotides from dNTPs mix. The number of DNA copied doubles every cycle because the newly formed DNA can serve as a template in the next cycle [38]. These steps represent one cycle which is usually repeated 30 times.

Some DNA polymerases need heat activation in the beginning so an initialization step is included in which the thermocycler is heated to around 95 °C before the thermocycling starts. The final elongation step (around 70 °C) is also usually performed to ensure that any remaining single stranded DNA is fully extended after the last PCR cycle, as well as the final hold step at 4 °C [36, 37]. PCR product is then checked using gel electrophoresis [38].

2.5 Gel electrophoresis

In general, gel electrophoresis is an analytical and separation method used to evaluate proteins or nucleic acids according to their size and/or charge in the electric field [40]. It is also the easiest way how to check the PCR product. The range of DNA fragments that can be separated ranges from 100 bp to 25 000 bp [41]. Since DNA is a negatively charged biomolecule due to the phosphate backbone, it has a constant charge-to-mass ratio unaffected by the size of the molecule, and so DNA molecules can be sieved by the gel matrix only by the size [42].

Samples are loaded into the gel matrix and after applying the electric field, negatively charged DNA molecules are repelled from the negative cathode and migrate towards the positive anode. Gel matrix serves as a sieve so migrating molecules are separated according to their size. Shorter molecules migrate more easily through the pores so they move faster; according to the phenomenon called sieving [40]. The rate of migration is influenced for example by the size of DNA molecule (the shorter the faster), agarose concentration (usually around 1% w/v), applied voltage (usually constant voltage [43], lower voltage gives better resolution but it takes more time), DNA conformation and electrophoresis buffer [41]. Commonly used buffers are TAE (Tris, acetate and EDTA) or TBE containing borate instead of acetate. TBE is used for long runs to prevent overheating because it is a better conductive medium [43]. Different intercalating DNA stains can be used to visualize DNA fragments. Previously used toxic ethidium bromide was replaced by GelRed or GelGreen. These stains bind to the DNA and provide visualization upon illumination. There also two staining methods for agarose gel, either DNA staining by post gel staining or DNA staining during the gel preparation [44]. The DNA can also be stained during sample preparation.

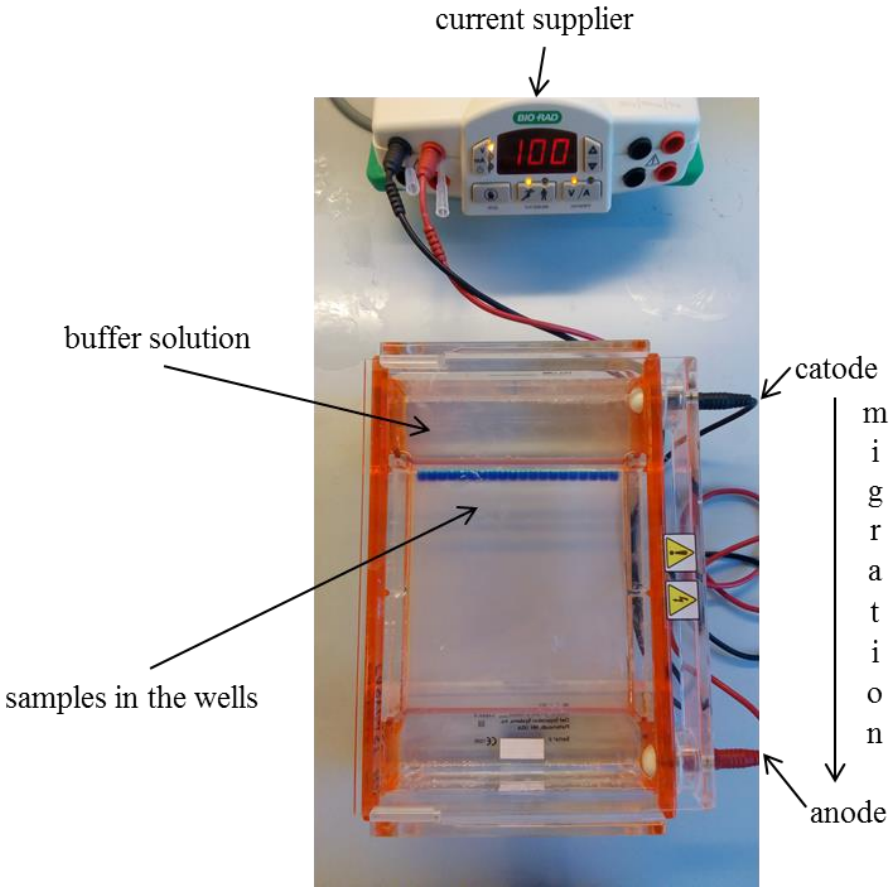

Figure 6: Gel electrophoresis instrumentation.

2.5.1 Electrophoresis mobility shift assay (EMSA)

The electrophoretic mobility shift assay (EMSA), also known as gel retardation assay or band shift assay, was originally used for detection of interaction between DNA and proteins [46] but can be also used to detect DNA-surfactant interaction [47]. EMSA is a type of non-denaturing electrophoresis in polyacrylamide or agarose gel and therefore the migration ability in the gel is proportional to the size of DNA [48]. When subjected an electric field,

the DNA-surfactant complexes migrate in the gel more slowly than the free DNA. This difference causes a change in DNA mobility in the gel band when compared with free DNA [49]. DNA can be labelled either by radioisotope probe ^{32}P or by fluorescent dye (which gives lower sensibility). The main advantages of EMSA are simplicity, robustness, ability to accommodate a wide range of conditions, and the very high sensibility even at very low volume [46] (20 μL, DNA concentration 10^{-12} M [51]), which even allows detecting conformational changes [49]. It is also pretty fast method which does not required very sophisticated instrumentation [48].

2.5.2 DNase protection assay

DNase protection assay, sometimes also called DNase footprinting, is a molecular method developed by Galas and Schmitz in 1978 to identify the sequence-specific binding of small molecules, oligonucleotides or proteins to DNA [52]. It can also help to specify the binding and the structure on the protein-DNA complex [53]. The technique is based on the ability of ligand to protect DNA from enzymatic or chemical cleavage at their binding sites [52]. Since enzymatic cleavage is used in this work, chemical cleavage will not be mentioned in detail. The bound species protects the phosphodiester backbone of DNA from DNaseI-catalyzed hydrolysis in and around the binding site [54]. Such protection is granted because of steric hindrance, DNase is a protein of a size 4 nm in diameter [55]. Hence, the footprint gives a broad indication of the binding site, generally 8–10 base pairs (bp) larger than the site itself [54].After digestion, samples are evaluated by electrophoresis, followed by autoradiography (labelled DNA by radioisotope) or by UV imaging (fluorescent dye used).

2.6 Luminescence

Emission of the light from any substances is called luminescence and occurs from electronically excited states. There are several different types of luminescence depending on the excitation source. For example chemiluminescence (chemical reaction as a source) with special kind bioluminescence in living organism, photoluminescence (light), thermoluminescence (heat) etc. Two major categories of photoluminescence are fluorescence and phosphorescence [56]. Fluorescence refers to the emission of light by sample that has absorbed light but, in contrast to phosphorescence, fluorescence ceases to glow immediately after switching off the radiation source [57].

In general, there are two different excited electron states: singlet and triplet. Singlet state is a molecular electronic state in which electron spins are paired. This state is allowed and preferred. In comparison triplet states is energetically forbidden and molecules must first undergo spin conversion to produce unpaired electron, which is highly unfavourable process with low probability.

2.6.1 Fluorescence – theory, lifetime, quantum yield

The fluorescence process can be divided into three subsequent steps – excitation, excited state and emission. The diagram describing the steps is called Jablonski diagram and is show in *Figure 7*.

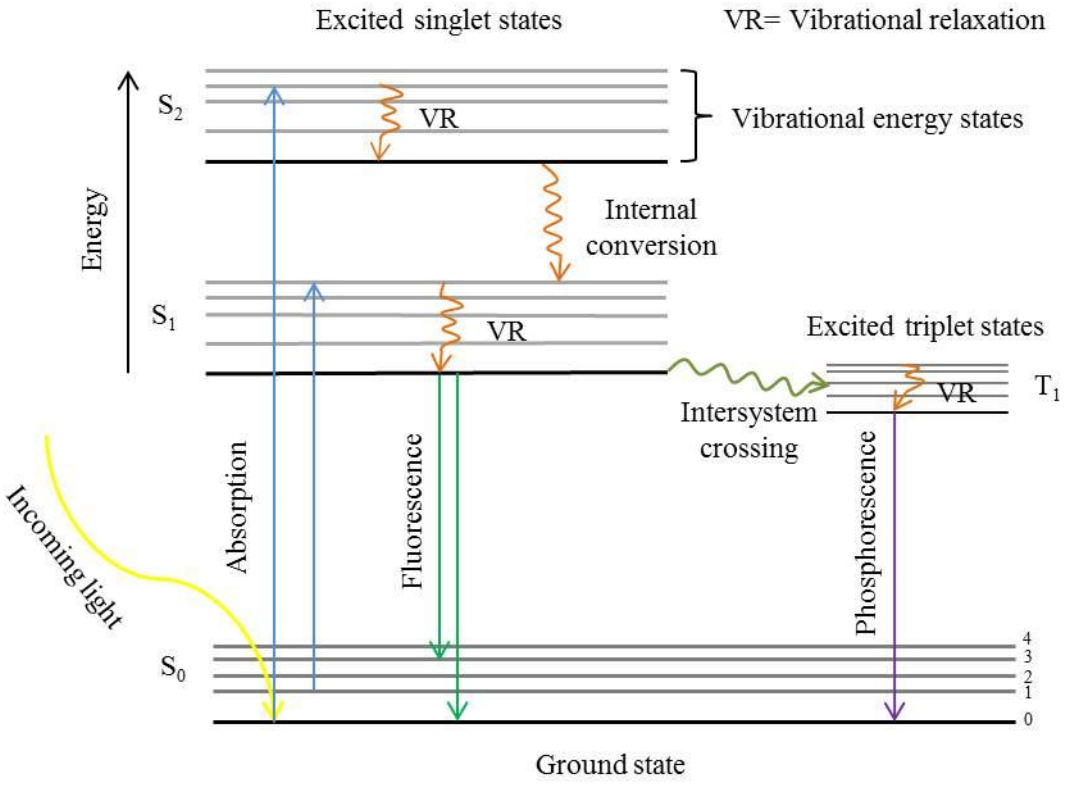

Figure 7: Jablonski Diagram.

Firstly, during excitation, a photon of energy is supplied by an external source (lamp or laser) and absorbed by fluorophores (molecules which are able to absorb and emit light) and going to the excited electronic singlet state S_1. Then, in the excited state which is very short, typically 1–10 ns, the fluorophore undergoes vibrational and conformational changes with two important consequences. First, the energy of S_1 is partially dissipated, yielding a relaxed singlet excited state S_1 from which the fluorescence emission originates. Second, other processes such as collisional quenching, fluorescence resonance energy transfer and intersystem crossing may occur, meaning that not all molecules initially excited by absorption return to the ground state S_0 by fluorescence emission. In the last step, the photon is emitted and the fluorophore returns to its ground state S_0. The energy of this photon is lower, and therefore the wavelength of the emission is longer, than the excitation photon, due to energy dissipation during the second phase [56]. The difference in the energy or wavelength is called Stokes shift (*Figure 8*).

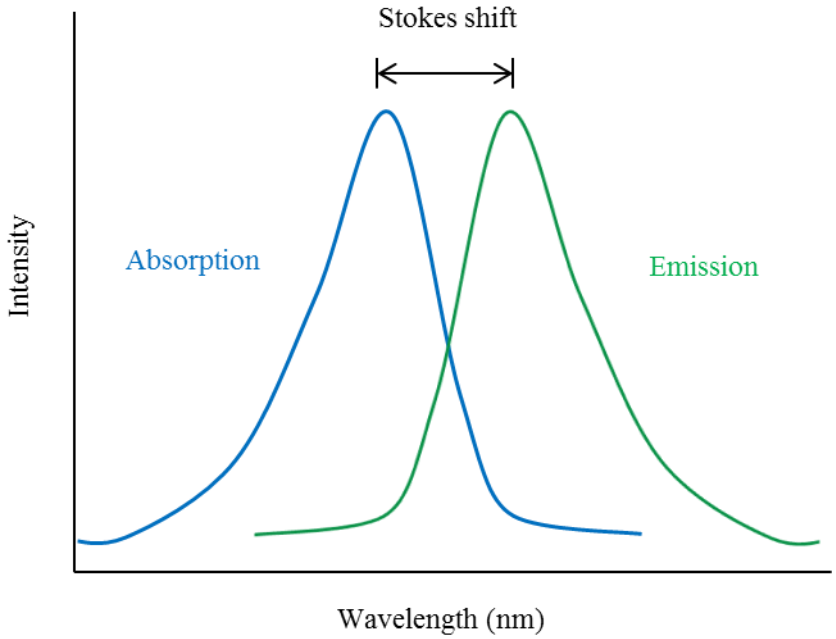

Figure 8: Stokes shift.

The lifetime (τ) of the fluorophore is defined as an average time between its excitation and return to the ground state, for fluorescence this is typically very short, 10 ns, in contrast to phosphorescence where the life time is in the range of milliseconds to seconds.
Quantum yield (ϕ) is defined as a ratio between the number of fluorescence photons emitted and a number of photons absorbed [58].

2.6.2 Emission and excitation (absorption) spectrum

The entire fluorescence can be cyclical process until the fluorophore is irreversibly destroyed (photobleached) in the excited state. Every fluorescent molecule has two characteristic spectra – excitation and emission.

Excitation spectrum is a dependence of fluorescence intensity on wavelength at constant emission wavelength.

Emission spectrum is a dependence of fluorescence intensity within a certain wavelength range at constant excitation wavelength. As mentioned before, emission occurs when the electron, which was excited to the higher energy state, goes down to the lower energy level, which can be accompanied by emission on the light. The wavelength of the photon is determined by the energy difference between these two states. Emission spectrum is usually mirror symmetry of excitation spectrum due to the fact that absorption and emission from the corresponding vibrational level have the same relative probability. Emission spectrum is unique and can be used for example to identify the elements in unknown substances, by spectroscopy technique [58].

2.6.3 Fluorescence spectroscopy

Fluorescence spectroscopy is widely used in chemistry, and biophysics due its high sensitivity and selectivity. It allows detailed, real-time observation of the structure and dynamics of intact biological systems [59]. This method uses special molecules containing fluorophores, which allows locating and imaging specific targets. Usually during

the measurement, fluorescent emission and absorption of the sample is measured, meaning that excitation wavelength is fixed [57].

Fluorescence spectroscopy analyses fluorescence from the sample and the device measuring fluorescence is called fluorimeter which is composed of incident photon source (xenon lamp for example) and monochromator used for selecting particular wavelength, sample holder, photon collecting detector (single or preferably multiple channel) and control software. So the light (from the excitation source) passes through a monochromator to the sample. A part of the incident light is absorbed by the sample leading to the sample fluorescence. The fluorescence light is emitted in all directions but part of this fluorescence light passes through the second monochromator and reaches the detector. The detector is placed at 90° to the incident light beam so it minimizes the chance of the transmitted or reflected incident light to reach the detector [59].

2.6.3.1 Dye exclusion assay

Dye exclusion assay is a method based on the changes in the fluorescence intensity. These changes are caused by exclusion of the fluorescent nucleic acid stain, for example Gel Star or ethidium bromide, which was originally bound to DNA [68]. Nucleic acid stain binds to DNA when it is in its extended conformation. So, it is possible to follow these changes by steady state fluorescence measurements, because the stain is expelled from the DNA when it undergoes conformational changes from extended to compacted state. Since Gel Star has much stronger fluorescence emission when bound to DNA than when free in solution [60], the emitted fluorescence is linearly dependent on the concentration of DNA in the extended conformation. Dye exclusion can be caused, for example, by adding DNA-binding or crowding agents [28].

2.7 Measurement of the critical micellar concentration

As already mentioned, critical micellar concentration (CMC) is the concentration at which the single monomeric units of surfactant aggregate and form larger structures called micelles. The CMC value is characteristic for each surfactant at given temperature. There are several techniques to measure CMC, the most common is tensiometry, but self-diffusion measurements, NMR or fluorescence spectroscopy can also be used. High resolution ultrasonic spectroscopy (HRUS) is a novel approach to determine CMC [61].

2.7.1 Tensiometry

Tensiometry is well known technique for surface tension measurement (γ) of liquids or surfaces. There are several different methods used for surface tension measurements, the oldest, capillary rise method [31], Wilhelmy plate method elaborated by Ludwig Wilhelmy [34], Du Noüy method [44] or maximum bubble pressure method [50]. The theory of these methods will not be discussed here since the measurement was used just shortly and performed by Dr. Jitka Krouská.

2.7.2 High resolution ultrasonic spectroscopy (HRUS)

HRUS is a pretty new technique for material analysis, which possesses many advantages. The sample does not have to be transparent because the ultrasonic waves propagate through the opaque system as well as transparent system and concentrated dispersions. It is non-destructive method extremely sensitive to intermolecular interactions which does not require any markers. Another important advantage is small volume of the sample; it is possible to

measure in the cell of 30 μL, which is very useful when working with bio material. HRUS can be used to measure chemical reaction, composition analysis, conformational transitions in materials, aggregation and gelatinisation, crystallisation, particle and droplet sizing, phase transition and measurements of the CMC, which is used in this work.

In general, it measures the velocity and attenuation of sound waves at high, ultrasonic frequencies propagating through the material. The ultrasonic velocity is mainly measured as an elasticity change in the sample. When the ultrasonic wave travels through the sample, the molecules respond to the oscillation deformations through inter-molecular repulsive (compression) and attractive (decompression) forces. The second measured parameter, attenuation, is measured as a loss of energy of the ultrasonic wave and scattering process. It is measured as the reduction in amplitude of an ultrasonic wave which has travelled a known distance through a medium per unit travelled distance.

Standard measurement is performed as a simultaneous comparison of reference cell with pure solvent and cell with the sample. In this case, the arising micelles are investigated by titration of surfactant solution to solvent in one cell and compared to second cell with pure solvent. Ultrasonic velocity is measured as it decreases with arising micelles. Hydrophobic core is elastic which means that solution with micelles is more elastic than pure solvent. The ultrasonic speed as a function of surfactant concentration is evaluated. There is a linear increase of the ultrasonic velocity before micelles are formed. When the micelles are formed, the ultrasonic velocity remains constant.

3 Material and methods

3.1 Materials

Water used in all experiments – ultrapure Milli-Q

3.1.1 PCR

Taq PCR kit – standard Taq reaction buffer, 10 mM dNTPs mix, forward primer A, reverse primer B, Taq DNA polymerase
plasmid – pSB-E1g from Escherichia Coli, 4017 bp

3.1.2 Gel electrophoresis

0.8% (w/v) Agarose with DNA stain GelRed™
1× TAE buffer – Tris, acetic acid, EDTA and MiliQ-water
Gel Loading Dye, purple (6×)
Quick-Load® Purple 1 kb DNA Ladder

3.1.3 DNA Purification

DNA Clean & Concentrator™-5 kit – DNA Binding buffer, DNA Wash Buffer, DNA Elution Buffer, Zymo-Spin™ Column[2], Collection tube

3.1.4 Dye exclusion assay

100× Gel Star™, fluorescent dye
Cetyl trimethylammonium bromide (CTAB)
Polyethylen glycol (PEG)
Polyacrylic acid (PAA)
Sodium bromide (NaBr)
Tris-HCl

3.1.5 EMSA

0.8% (w/v) Agarose with DNA stain GelRed™
1× TAE buffer – Tris, acetic acid, EDTA and MiliQ-water
Cetyl trimethylammonium bromide (CTAB)
Polyethylene glycol (PEG)
Sodium bromide (NaBr)
Tris-HCl
DNase

3.1.6 CMC measurements

Cetyl trimethylammonium bromide (CTAB)
Polyethylen glycol (PEG)
Sodium bromide (NaBr)
Tris-HCl

3.2 DNA preparation

3.2.1 Polymerase chain reaction (PCR)

The sample for PCR was prepared according to the protocol [19]. The total volume of the sample was 50 μL. Firstly, 40.6 μL nuclease free water was pipetted into an Eppendorf tube. Then 5μL 10× standard *Taq* Reaction Buffer (final c 1×), 1μL 10 mM dNTPs (final c 200 μM), 1 μL 10μM Forward Primer (final c 0.2 μM), 1 μL 10 μM Reverse Primer (final c 0.2 μM), 1 μL DNA Template, 0.4 μL *Taq* DNA Polymerase was added. Sample was carefully mixed with pipette; drops on the walls were removed by a quick centrifugation run. Components were stored in the cooling block during preparation; samples were stored in the ice.

Cycle conditions were set up as follows. Initial denaturation at 95 °C for 30 s one cycle, then 35 cycles composed of denaturation at 95 °C for 25 s, annealing at 55 °C for 20 s and extension at 68 °C for 1 min. The final extension is one cycle at 68 °C for 5 min. After this, samples were held at 4 °C until withdrawal. Samples were kept in the freezer at −27 °C.

3.2.2 Gel electrophoresis

For checking the number of DNA base pairs, gel electrophoresis with 0.8% (w/v) agarose and GelRed was used. 1× TAE (Tris-acetate EDTA) was used as a buffer. Mixture of 7 μL MQ-water, 1 μL PCR product and 2 μL of loading dye (final concentration 1×) was loaded into the gel. Mixture of 7 μL MQ-water, 1.5 μL GeneRuler™ 1 kb DNA Ladder and 2 μL of loading dye were loaded as a control. The Bio-Rad PowerPack™ Basic Power Supply was set up at 100 V and run for 30 min. The Bio-Rad GelDoc™ was then used to image the gel.

3.2.3 DNA purification

DNA Clean & Concentrator™-5 kit was used to purify DNA. PCR product was mixed with DNA Binding Buffer in a 1:5 volume ratio. Mixture was loaded into the Zymo-Spin™ Column^2 in a Collection Tube and centrifuged at high speed (≥ 10 000 RCF) for 30 s, the flow-through was discarded. 200 μL DNA Wash Buffer was added to the column and centrifuge at the same speed for 30 s, the procedure was repeated. The Zymo-Spin™ Column^2 was placed into a new 1.5 mL Eppendorf Tube, 30 μL DNA Elution Buffer was added directly to the column matric and incubated at room temperature for one minute, finally, a final round of centrifugation at high speed for 30 s allowed to elute the DNA. The pure DNA solution was stored in the freezer (−27 °C).

3.2.4 DNA concentration

DNA concentration was measured by Thermo Scientific NanoDrop ND-1000 Spectrophotometer in Nucleic Acid mode. Nuclease free water (1 μL) was loaded on the lower optic surface to initialize the spectrophotometer and cleaned. Blank measurement was performed with 1 μL nuclease free water and cleaned. To measure DNA concentration, 1 μL DNA sample was loaded on the lower optic surface. Once the measurement was complete, the surface was cleaned with nuclease free water and cleaned.

3.3 Dye exclusion assay

3.3.1 Sample preparation for PEG and CTAB control samples

For sample preparation Thermo Fisher Scientific Nucleon 96 well flat black plates were used. The total volume of sample was 50 µL. Samples were prepared as shown in Table 1 in Appendix, different salt conditions were used.

Stock solutions were prepared 10× more concentrated and 5 µL of each stock solution was pipetted. Gel Star™ (100×) was prepared from 10 000× stock solution by 100× dilution with Milli-Q water. DNA (20 mg/mL) working stock was prepared from main stock by dilution with Milli-Q water. NaBr (100/500/1 000 mM) working stock was prepared by weighing solid NaBr and mixing with aliquot volume of Milli-Q or Tris buffer (100/200 mM) depending on desired conditions. Different PEG concentrations were prepared by dilution of 400 mg/ml main stock with aliquot volume of Mili-Q water. Different CTAB concentrations were prepared by dilution of main CTAB stock (1 mM) by Tris (10 mM).

Firstly, equal volumes of Gel Star™ (100×) and DNA (20 mg/mL) were pipetted, thoroughly mixed and incubated for 15 minutes. Aliquot volume of 10 mM Tris buffer was added. Then, depending on either PEG or CTAB investigation, 5 µL of either PEG or CTAB (increasing concentration) was added, thoroughly mixed and incubated for either 30 minutes (PEG) or 60 minutes (CTAB).

New pipet tip was used per each pipetting to avoid stock solution contamination. Samples were thoroughly mixed up and down with pipet.

3.3.2 Sample preparation for molecular crowding research

For sample preparation Thermo Fisher Scientific Nucleon 96 well flat black plate was used. The total volume of samples was 50 µL. Stock solutions (10× higher concentration) of each component were prepared and 5 mL of each was pipetted as follows. Different salt and buffer conditions were investigated; suitable conditions were chosen as follows.

Firstly, Gel Star™ (100×) and DNA (20 mg/mL) were pipetted, thoroughly mixed and incubated for 15 minutes. After incubation, aliquot volume of 10 mM Tris and 5 µL CTAB of different concentrations were added. Salt effect was studied by adding 5 µL 100 mM NaBr. Samples were thoroughly mixed and incubated for 60 minutes. Crowding was mimicked by adding 5 µL of 250 mg/mL PEG or PAA. Samples were thoroughly mixed and incubated for 30 minutes. Detailed description can be found in Appendix (Table 2–7).

3.3.3 Instrument setup

Tecan Infinite M200 PRO multifunctional plate reader was used for steady state fluorescence spectra measurements. Firstly, Thermo Fisher Scientific Nucleon 96 well flat black plate was shaked for 30 s with 1.5 mm amplitude and orbital type of shaking. Then, fluorescence scan in bottom mode was performed. Excitation maximum of Gel Star™ was set up as 485 nm and emission at 527 nm. Gain was set up as manual and equal to 70. Circle multiple reads per well was used with size 3×3 and 500 µm border. In the end, fluorescence intensity scan was obtained. Emission scan mode was used with excitation wavelength 485 nm and emission from 500 to 700 nm with 1 nm step. Gain was manual and equal to 70.

3.4 Electrophoresis mobility shift assay (EMSA)

3.4.1 Sample preparation

The total volume of the sample was 20 μL. Both ionic strength and crowding effects were investigated. Samples were prepared by pipetting 3 μL DNA (33 mg/ml), aliquot amount of 10 mM Tris, 2 μL CTAB (increasing concentration). Salt effect was investigated by adding 2 μL NaBr (1 000 mM), after mixing and 60 minutes incubation, crowding environment was introduced by adding 2 μL PEG (250 mg/mL) and samples were thoroughly mixed and incubated for 30 minutes. Protocol is described in Appendix (table 8–11).

3.4.2 Instrument setup

For EMSA measurement, 0.8% (w/v) agarose gel with GelRed was used. The Bio-Rad PowerPack™ Basic Power Supply was set up at 40 V for the first 30 min and then at 90 V.

3.5 DNase protection assay

3.5.1 Sample preparation

Samples were prepared in a similar way as for EMSA, but the final volume of the sample was 50 μL. Working DNA stock solution (50 mg/mL) was prepared from the main stock by dilution of aliquot volume of Mili-Q water. Different CTAB concentrations were as described in the Appendix (Table 12). Different salt conditions were investigated. As a low salt condition, 10 mM Tris was used, 100 mM NaBr for high salt conditions, details are described in Appendix (Table 13). PEG working stock solution (250 mg/mL) was prepared from the main stock (400 mg/mL) by dilution of Mili-Q water. After incubation, 5 μL of DNase buffer and 1μL of DNase enzyme was added and thoroughly mixed with the pipette. The plate was incubated for 20 min at 37°C. After incubation, 6 μL of 6× loading dye was pipetted and mixed, then 10 μL of the sample was loaded into the 0.8% (w/v) agarose gel with GelRed. The gel was screened every 30 min during the electrophoresis using the Bio-Rad GelDoc™.

3.5.2 Instrumental setup

For DNase protection assay measurement, 0.8% (w/v) agarose gel with GelRed was used. The Bio-Rad PowerPack™ Basic Power Supply was set up at 40 V for the first 30 min and then at 50 V.

3.6 CMC measurements

CMC measurements were performed at Material Research Centre in Brno by junior researchers, so it is mentioned just in brief. First, measurement of CMC was probed by classical method, tensiometry. Then, the new method for CMC determination – HRUS were used and CMC values were compared.

3.6.1 Tensiometry

Measurement was performed by Dr. Jitka Krouská using Tenziometr KSV Sigma 701. This measurement was performed to get general knowledge about behaviour of the system. The Samples with increasing CTAB concentration, 10 mM Tris and CTAB, 10 mM Tris and

25 mg/mL PEG and CTAB, Tris and 10 mM NaBr and CTAB, 10 mM Tris, 10 mM NaBr and 25 mg/mL PEG were prepared and surface tension was measured.

3.6.2 HRUS

Measurement was performed by Dr. Kargerová at Material Research Centre in Brno using spectrometer HR-US 102T. The effect of crowding agent on CMC value was studied as well as salt effect. Samples with increasing CTAB concentration, 10 mM Tris and CTAB, 10 mM Tris and 25 mg/mL PEG and CTAB, Tris and 10 mM NaBr and CTAB, 10 mM Tris, 10 mM NaBr and 25 mg/mL PEG were prepared and the ultrasonic velocity was measured. The reference cell filled with corresponding sample without CTAB, depending on the measurement.

4 Results and discussion

4.1 PCR and gel electrophoresis

DNA used is this work was produced by PCR using protocol which was optimized in previous work by Sravani Ramisetty. pSB-E1g plasmid was used as a template according to the previous work.

PCR product was checked by gel electrophoresis. *Figure 9* shows GelDoc™ image of 0.8% agarose gel with PCR product. First lane shows the DNA ladder, and the PCR product is compared in the following lanes. In order to confirm desired product, only one bright band has to be present. In addition, the band has to correspond to a DNA product with the same bp size (pSB-E1g contains 4017 bp), as checked against the ladder. The dark intensive band means that the DNA is highly concentrated.

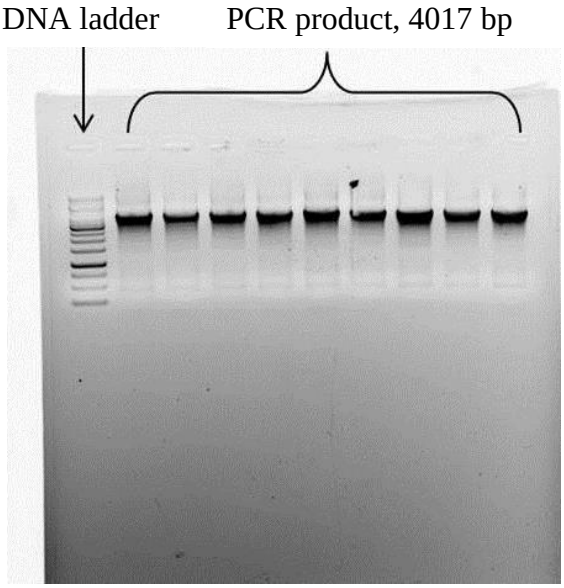

Figure 9: Electrophoresis of PCR amplified fragments.

4.1.1 DNA concentration measurement

Thermo Scientific NanoDrop ND-1000 Spectrophotometer was used in Nucleic Acid mode to measure DNA concentration. In order to confirm DNA purity two ratios are taken into account. .DNA is free from contaminants if the 260/280 ratio is around 1.8–2. Appreciably lower ratio indicates contaminants in the sample, such as proteins, phenol or other contaminants absorbing at 280 nm. The 260/230 ratio for "pure" DNA is commonly in the ange 2.0–2.2. Lower values indicate contaminants absorbing at 230 nm. These contaminants may be residual phenol from nucleic acid extraction, residual guanidine, which is usually used in column based kits or glycogen used for precipitation. *Figure 10* shows a DNA concentration of 165.9 µg/mL and that the sample is free of the most common contaminants. PCR and further concentration determination was carried out after every PCR protocol.

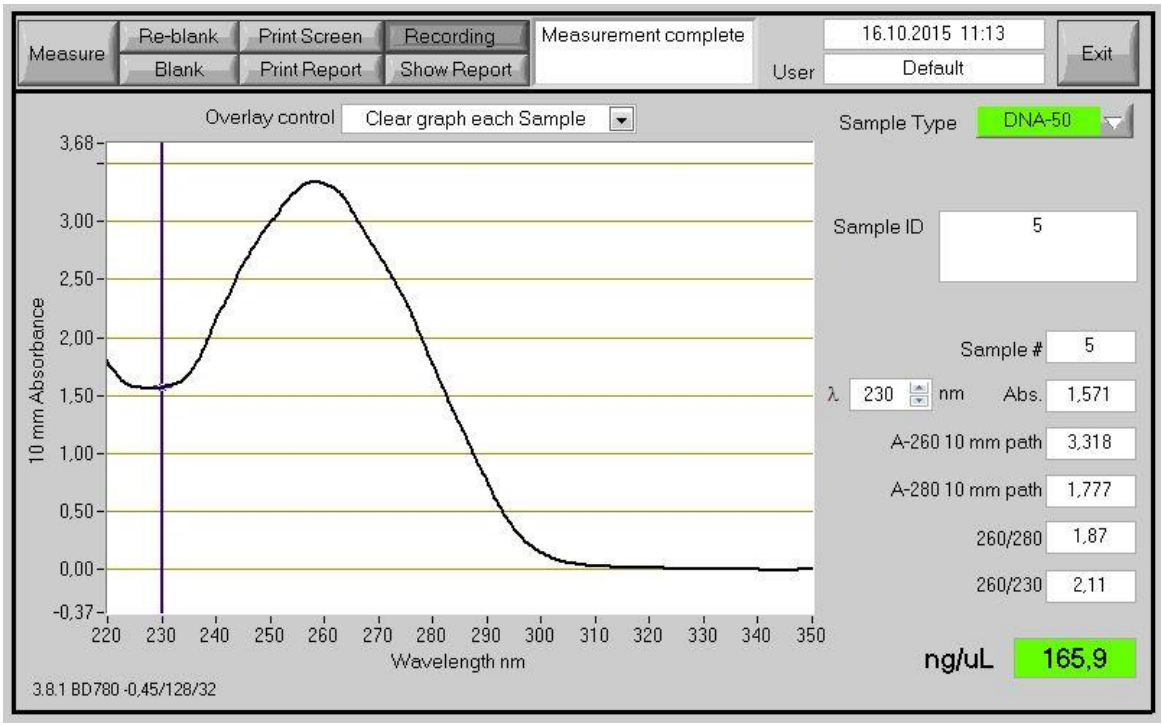

Figure 10: DNA concentration measurement using Nanodrop.

4.2 Dye exclusion optimization

Fluorescence intensity data were analysed using MS Excel. The data were normalized to the intensity of DNA alone. Investigation was made in different buffer conditions but the final buffer condition was chosen to be 10 mM Tris. Measurements of 2 or 3 independent sample sets were performed and error bars were calculated using MS Excel.

4.2.1 PEG research

PEG was chosen to mimic the crowding environment for its neutral charge and ubiquity in the living cells. Samples with constant DNA concentration and increasing PEG concentration in the presence of 10 mM Tris were investigated. Normalized fluorescence data are shown in *Figure 11*. Increasing PEG concentration leads to the decrease in fluorescence emission intensity, which correlates with DNA compaction and dye exclusion. Since PEG is a neutral molecule and does not bind to DNA, the dye exclusion is caused by DNA conformational changes induced by the excluded volume effect.

It has been found previously [28] that synergistic effects are more predominant when the individual crowding species (PEG and CTAB in this case) are present in low concentrations, which lead to none or only partial DNA condensation. Therefore, 25 mg/mL PEG concentration was chosen for following investigation. As it can be seen on the graph, 10 % of DNA is compacted at this concentration. Since PEG and PAA have approximately the same molecular weigth, the same concentration was used for studies involving PAA (25 mg/mL).

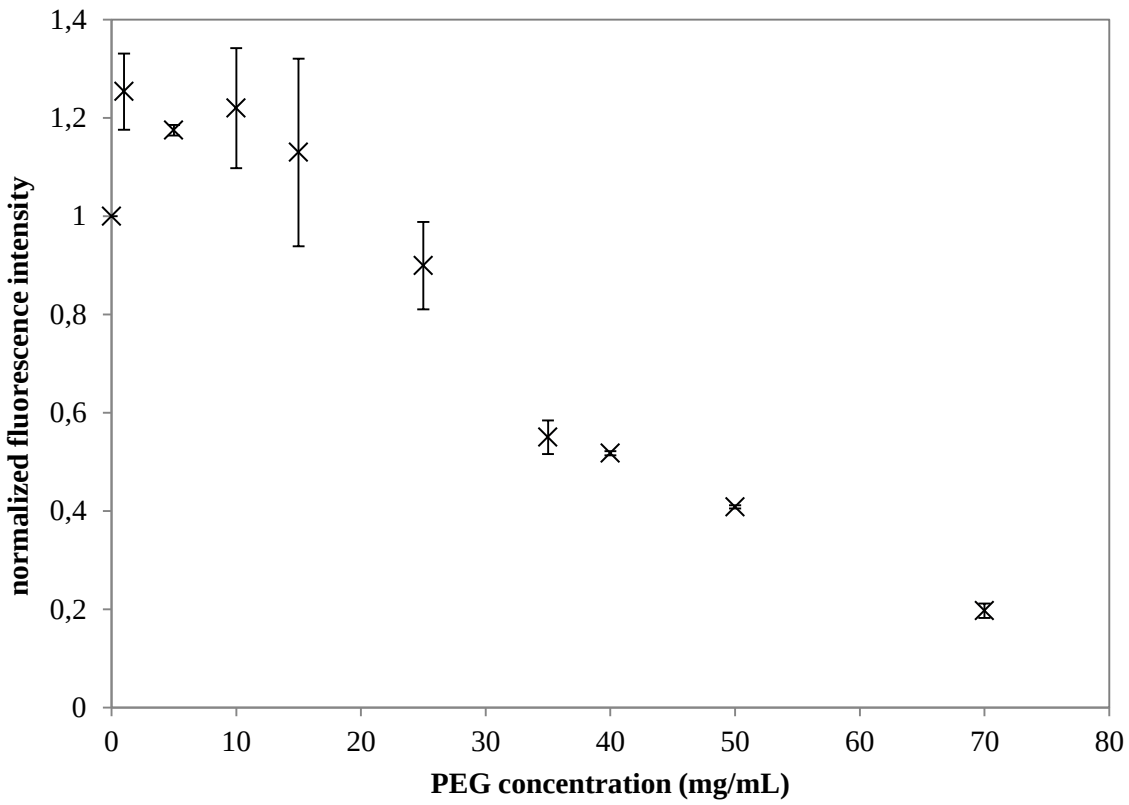

Figure 11: Fluorescence intensity of DNA-Gelstar complexes in 10 mM Tris, shown as a function of PEG concentration, normalized to the fluorescence intensity in the absence of PEG. The fluorescence emission was measured at 527 nm.

The influence of ionic strenght on DNA condensation by PEG was also investigated, and shows as normalized fluorescence data in *Figure 12*. Different NaBr concentrations were used – 10 mM, 50 mM and 100 mM. There was no significant ionic strenght effect in the range of 1 mg/mL up to 25 mg/mL PEG concentration. In case of chosen PEG concentration (25 mg/ml), the highest ionic strength was chosen for further investigation since it condenses the DNA a bit more so the salt effect can be investigated. The ionic strength effect is more significant for the higher PEG concentration. In comparison with previous PEG research, in higher PEG concentration, there is opposite effect than expected. DNA is less condensed than in case of no salt conditions. Polymer- and salt induced condensation usually promotes condensing of DNA but it this case, the retarding salt effect is obvious. This was mentioned by Yoshikawa and other in their work as mentioned in introduction [22]. This also correlates with Krotova and others results [23].

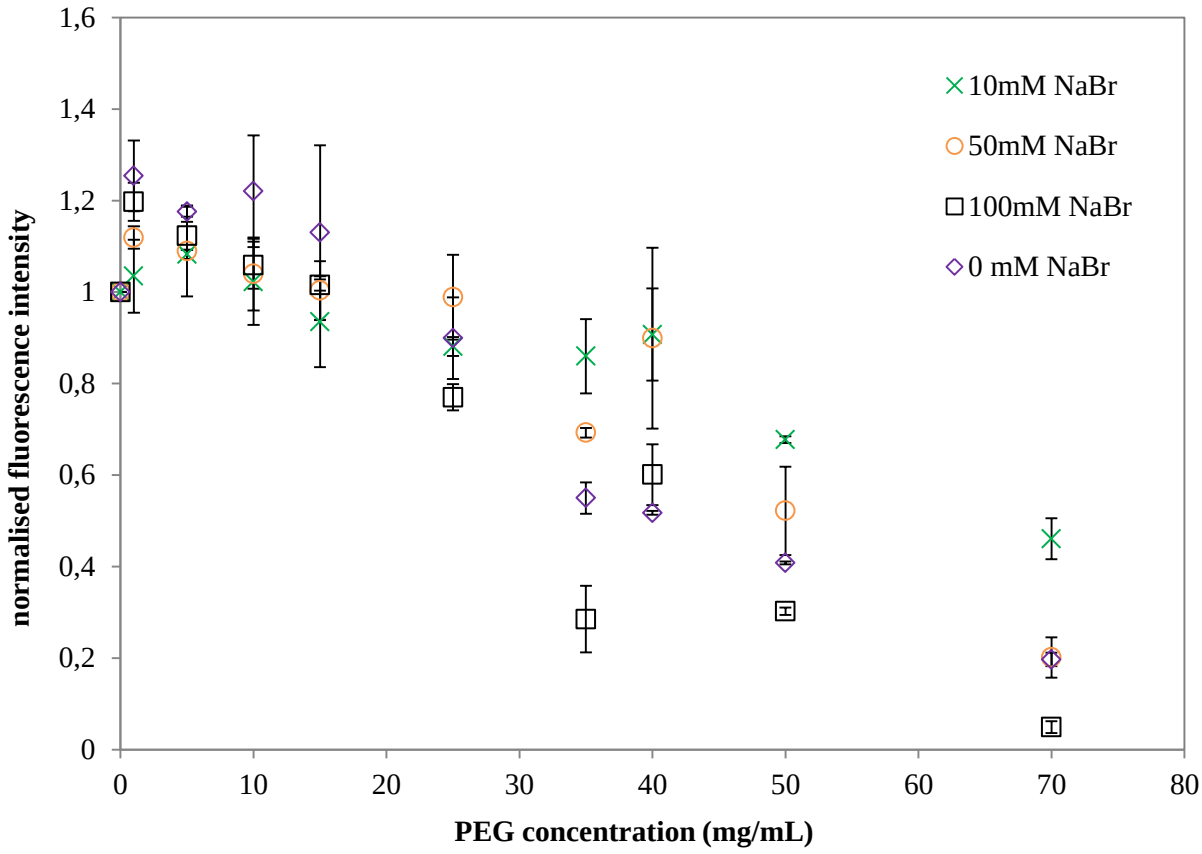

Figure 12: Fluorescence intensity of DNA-Gelstar complexes in 20 mM Tris, shown as a function of PEG concentration for different NaBr concentration, 10 (crosses), 50 (circles) and 100 (square) mM, normalized to the fluorescence intensity in the absence of PEG. The fluorescence emission was measured at 527 nm.

4.2.2 DNA condensation by CTAB

Systems with constant DNA concentration and increasing CTAB concentration in the presence of 10 mM Tris were investigated. It is obvious that DNA undergoes condensation very efficiently by the addition of CTAB. Higher CTAB concentration (50 μM and higher) condenses DNA totally.

Investigation was made in order to find the range of CTAB concentration where CTAB starts to compact DNA until it is completely compacted. Therefore, according to the normalized fluorescent data presented in *Figure 13*, we have worked with CTAB concentrations in the range of 5 to 100 μM in the work that follows, for 10 mM Tris buffer.

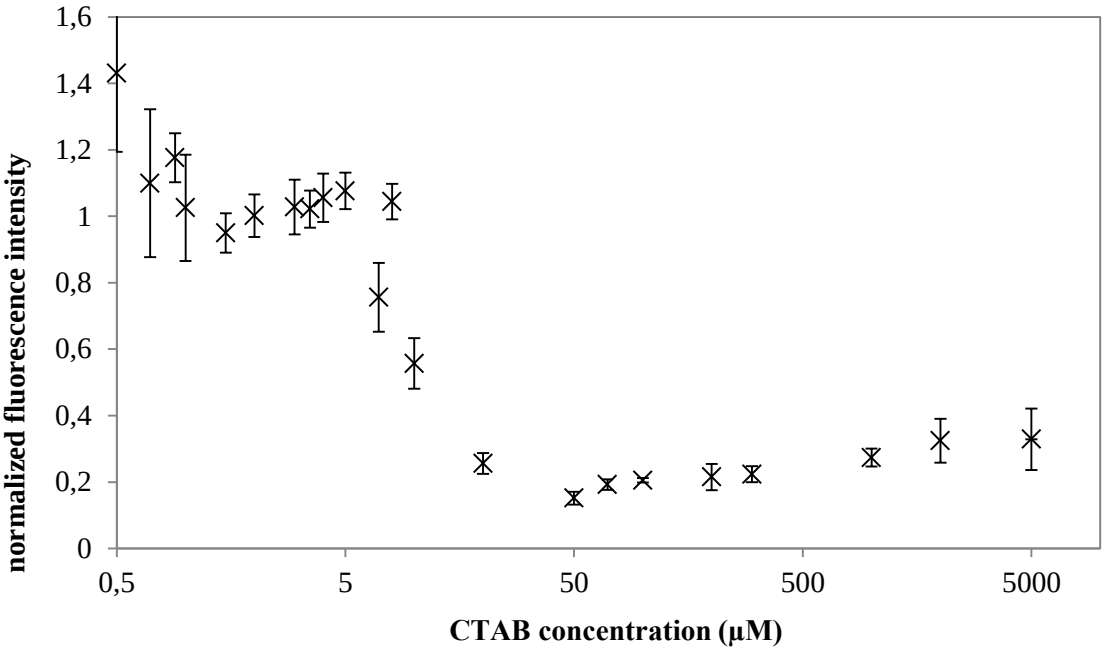

Figure 13: Fluorescence intensity of DNA-Gelstar complexes in 10 mM Tris, shown as a function of CTAB concentration, normalized on the fluorescence intensity in the absence of CTAB. The fluorescence emission was measured at 527 nm.

4.3 CTAB and PEG synergism in DNA condensation.

By using fluorescencent dyes that show an increased fluorescence emission when bound to DNA, steady state fluorescence can be used to study dye exclusion upon DNA condensation. In the compacted DNA state, the binding of the fluorophore Gel Star is hindered, meaning that the fluorescence is linearly dependent on the concentration of DNA in the extended conformation [28]. So in these experiments, it is possible to measure influence of PEG and NaBr on the DNA condensation.

In order to evaluate the crowding effect and the influence of ionic strength on DNA condensation by CTAB in the presence of PEG, samples composed of constant DNA concentration, 10 mM Tris, CTAB of increasing concentration, 25 mg/ml PEG and 100 mM NaBr were investigated.

The fluorescence intensity data normalized on the fluorescence intensity in the absence of CTAB and PEG (*Figure 14*) shows the molecular crowding effect. Fluorescence intensity decreases in both cases exponentially, even though there is an initial plateau in case of samples without PEG. The system is almost completely condensed for the samples with higher CTAB concentration (30 μM CTAB and higher) and therefore, above this concentration, there is no difference between samples with and without crowding environment. However, PEG addition slightly improves DNA condensation in the region where the DNA is not totally condensed just by CTAB (0-20 μM CTAB). Since the data was normalised to the intensity of DNA alone, system composed of Gel Star–DNA–PEG is presented in the graph bellow (orange circle with zero CTAB concentration). In this system, 90 % of DNA is not condensed which is in good agreement with the PEG investigation (*Figure 11*). For systems without PEG (crosses), the error bars are pretty large in the first third of the graph because the system is in the transition state from the extended form to

the condensed state. Therefore, it is not so easy to make conclusion for this region. This has been previously mentioned in DNA condensation studies [14, 62, 63].

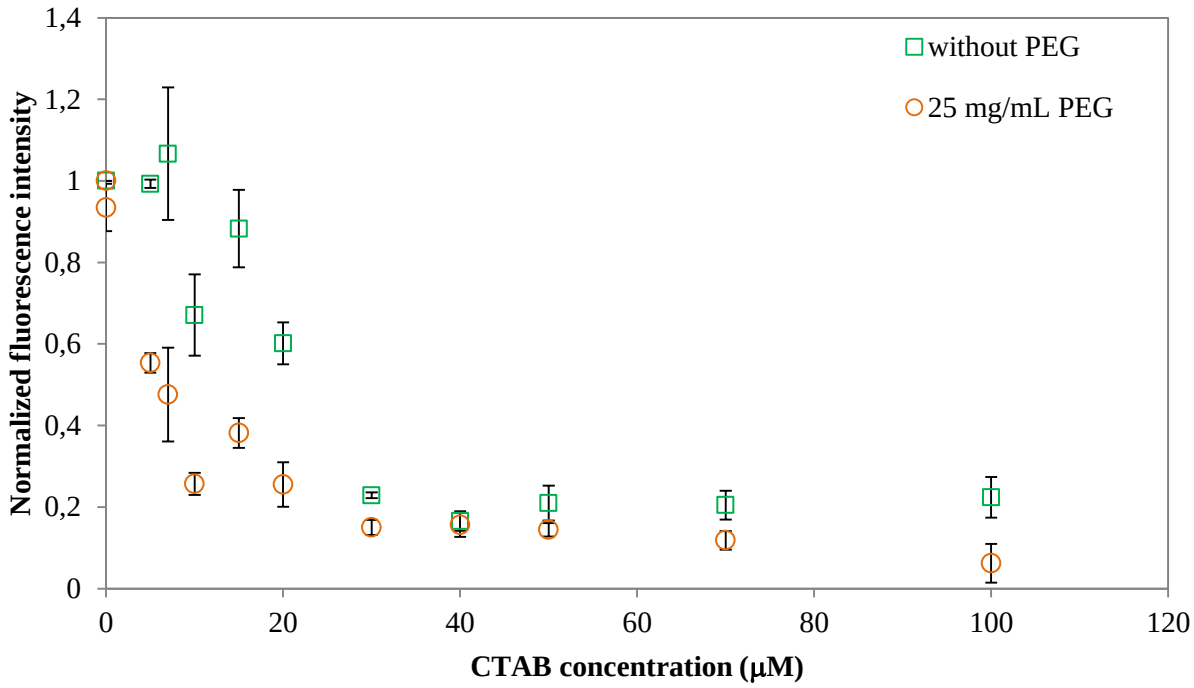

Figure 14: Fluorescence intensity of DNA-Gelstar complexes in 10 mM Tris, shown as a function of CTAB concentration for different concentration of PEG, 0 (squares) and 25 (circles) mg/mL, normalized on the fluorescence intensity in the absence of CTAB and PEG. The fluorescence emission was measured at 527 nm.

In case of ionic strength investigation, synergistic effect of NaBr and PEG is obvious, with the fluorescence intensity decreases significantly for the systems with crowding molecules (*Figure 15*). NaBr has significant effect on the DNA condensation in the region of low CTAB concentration in the presence of PEG. In the system with 5 μM CTAB, about 77 % of DNA is not condensed, but further addition of CTAB leads to a DNA condensation of around 75 %. This condensation is induced by synergistic effect of NaBr and PEG and is called polymer and salt induced (psi) condensation [6]. In the region of 40 μM and higher CTAB concentration, DNA is totally condensed due to high CTAB concentration since, as previously mentioned, higher CTAB concentration alone condenses DNA efficiently.

On the other hand, ionic strength has opposite effect on the samples without crowding molecules where the fluorescence intensity decreases almost linearly.

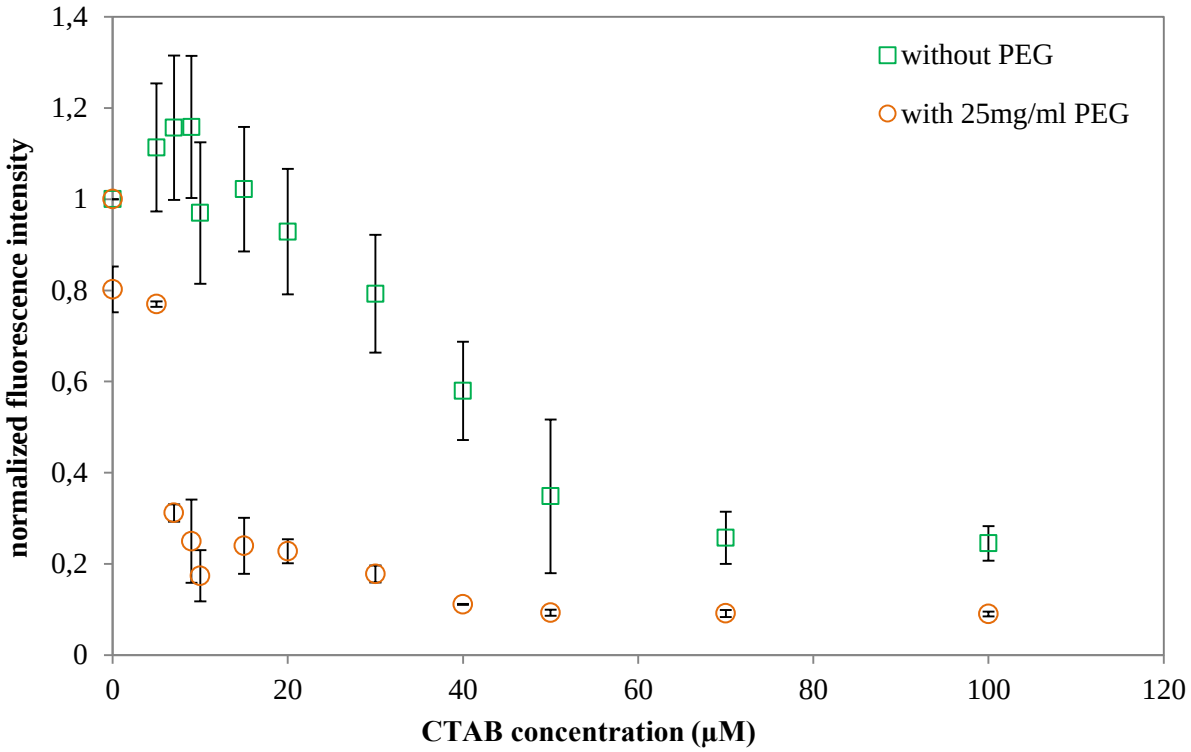

Figure 15: Fluorescence intensity of DNA-Gelstar complexes in 10 mM Tris and 100 mM NaBr, shown as a function of CTAB concentration for different concentration of PEG, 0 (squares) and 25 (circles) mg/mL, normalized on the fluorescence intensity in the absence of CTAB and PEG. The fluorescence emission was measured at 527 nm.

4.4 Competitive effect between CTAB and PAA in DNA condensation

For comparison, negatively charged PAA was used to mimic crowding environment. Experiment was performed in low and high salt conditions. Experiment followed the same conditions as previously but PEG was replaced with PAA. The fluorescence intensity data normalized on the fluorescence intensity in the absence of CTAB and PAA (low salt condition, 10 mM Tris) are showed in *Figure 16*. Since PAA is negatively charged, it can bind to CTAB through electrostatic interactions. The aim of this investigation was to find out whether CTAB prefers to bind to PAA or DNA.

It is obvious from *Figure 16* (blue circles) that CTAB prefers to interact with PAA. It means that DNA is present in the solution as an elongated coil leading to no decrease in fluorescence intensity upon increase in CTAB concentration. This is probably related with the larger flexibility of PAA compared to DNA. It is easier for the PAA to wrap around micelles.

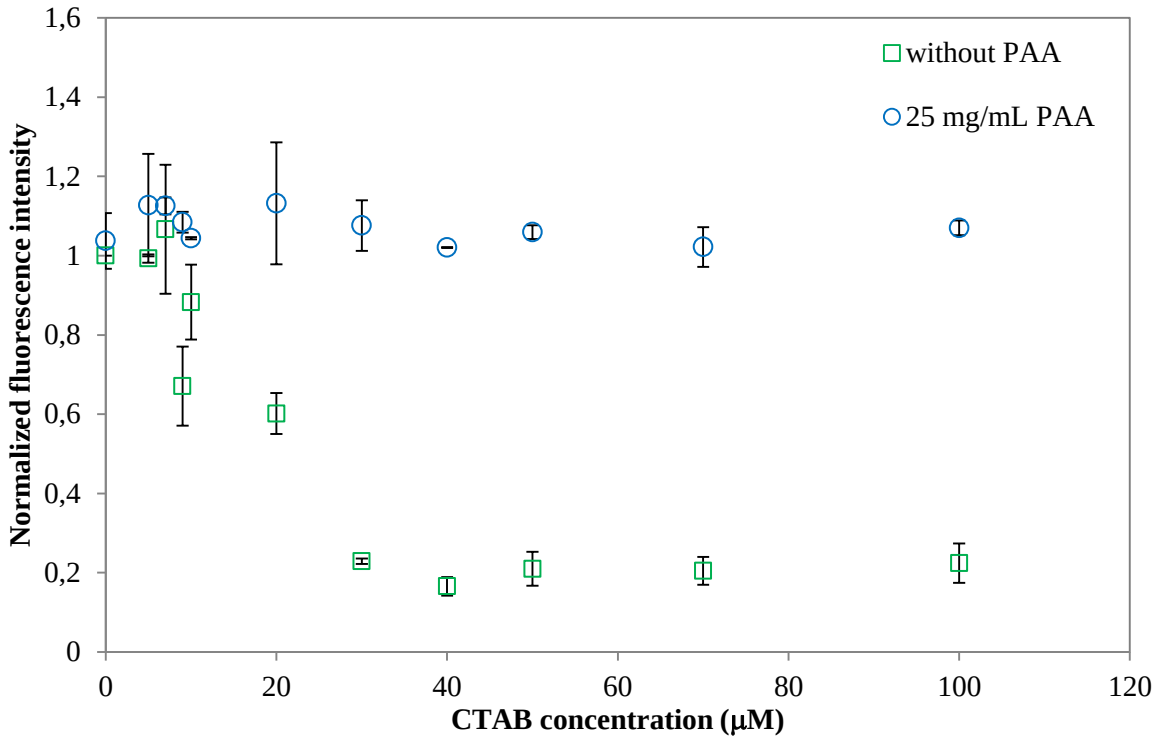

Figure 16: Fluorescence intensity of DNA-Gelstar complexes in 10 mM Tris, shown as a function of CTAB concentration for different concentration of PAA, 0 (squares) and 25 (circles) mg/mL, normalized on the fluorescence intensity in the absence of CTAB and PEG. The fluorescence emission was measured at 527 nm.

Such experiments were also performed in high salt condition, 100 mM NaBr, and the fluorescence intensity data normalized with the fluorescence intensity of DNA-GelStar complexes in the absence of CTAB and PAA are showed in *Figure 17*. It is clear from fluorescence intensity profiles that presence of salt and PAA influence DNA-CTAB interaction, in other words, DNA is not condensed at all. According to these results, no other experiment with PAA was performed (no DNA condensation).

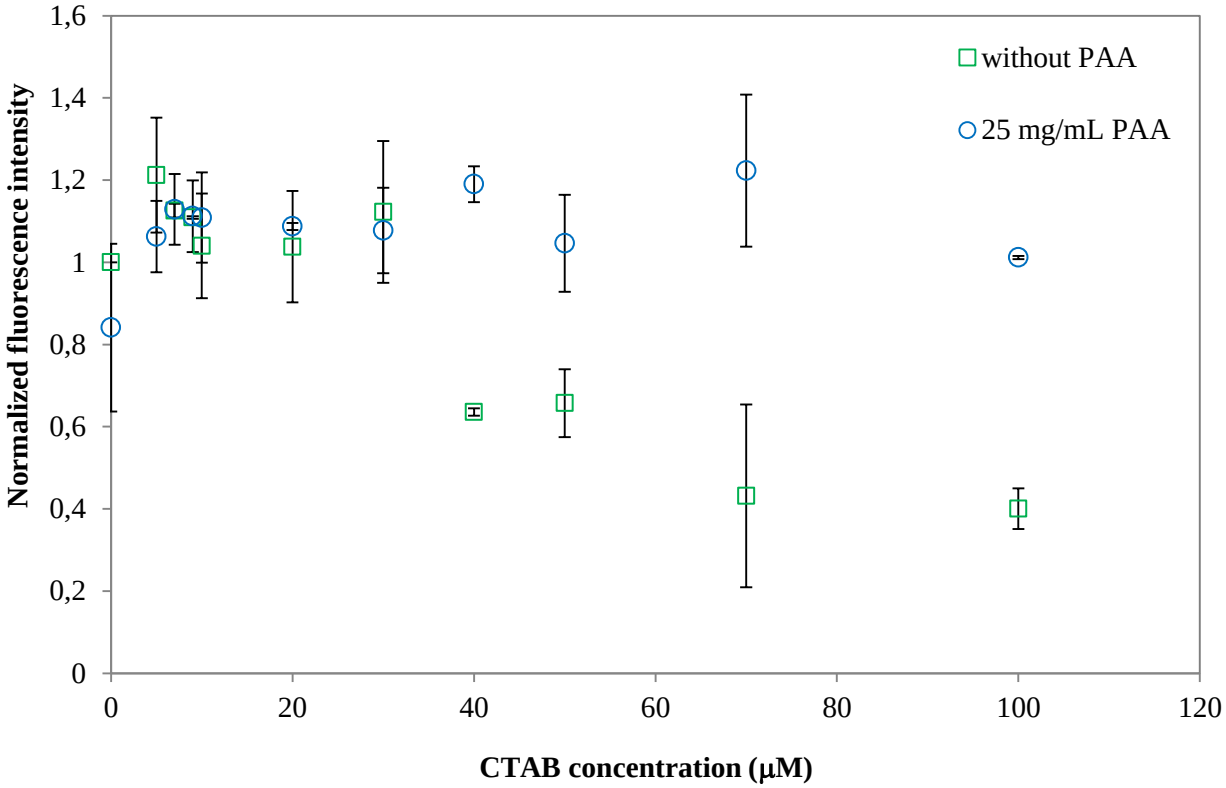

Figure 17: Fluorescence intensity of DNA-Gelstar complexes in 10 mM Tris and 100 mM NaBr, shown as a function of CTAB concentration for different concentration of PAA, 0 (squares) and 25 (circles) mg/mL, normalized by the fluorescence intensity in the absence of C CTAB and PEG. The fluorescence emission was measured at 527 nm.

4.5 EMSA

In order to compare the crowding effect and synergism of PEG on DNA–CTAB interaction and effect of salt, two different experiments were performed.

4.5.1 EMSA of DNA, CTAB, PEG systems at low salt conditions

Electrophoresis of the DNA-CTAB and DNA-CTAB-PEG systems was performed. The gel was checked after 30 min, 60 min and 100 min to compare if the complexes are moving at the same speed.

Although experiments were performed six times, two different results for samples with crowding environment were obtained (*Figure 18* and *Figure 19*).

In case of samples DNA-CTAB, no difference among measurements was observed. First six samples with increasing CTAB concentration (0–100 μM) move from the well and do not show any decrease in the band intensity, meaning that DNA concentration is approximately the same as in the first sample without CTAB. Sample in lane eight (200 μM CTAB) shows a decrease in the band intensity, meaning that DNA concentration is lower, suggesting that some of the DNA was retained due to complex formation, or that the DNA became inaccessible to the electrophoresis dye, also due to complex formation. Last two samples (500, 800 μM CTAB) underwent complex formation with no band, which means that every molecule of the DNA was involved in the arising complexes which were either neutrally

charged or too large to move. This fact was also visible by eye as a kind of blue complex in the well. In case of samples DNA-CTAB-PEG, in three independent investigations, a second band (*Figure **18***) appeared in the first six samples. The position of this second band is lower in the gel, meaning that the unidentified molecules are either of smaller size or have higher charge. It is hard to describe what could this band be, because there was no enzyme to cut the original DNA into the smaller parts. Also there is no possibility for DNA to become more charged just because of addition of PEG since we did not see this band in non-crowded samples. It should be noticed that the second band appeared even in the first sample with zero CTAB concentration. Second bands are more intense than the regular ones, which are almost invisible in case of samples with 5, 10, 20 μM CTAB. Then, for samples with 50 and 100 μM CTAB, two bands again appeared and for sample with 200 μM CTAB, only one band of 4000 bp size with low intensity appeared. In this sample, it was obvious by eye that the complex was formed and stayed in the well as well as for last two samples, 500 and 800 μM CTAB.
In case of the second results obtained, no second band appeared at all. The movement from the well for the samples in the crowding environment is similar to those for non-crowded environment; the only difference is in the band intensity, which means that PEG enhances complex formation so that some DNA is retained in the wells.

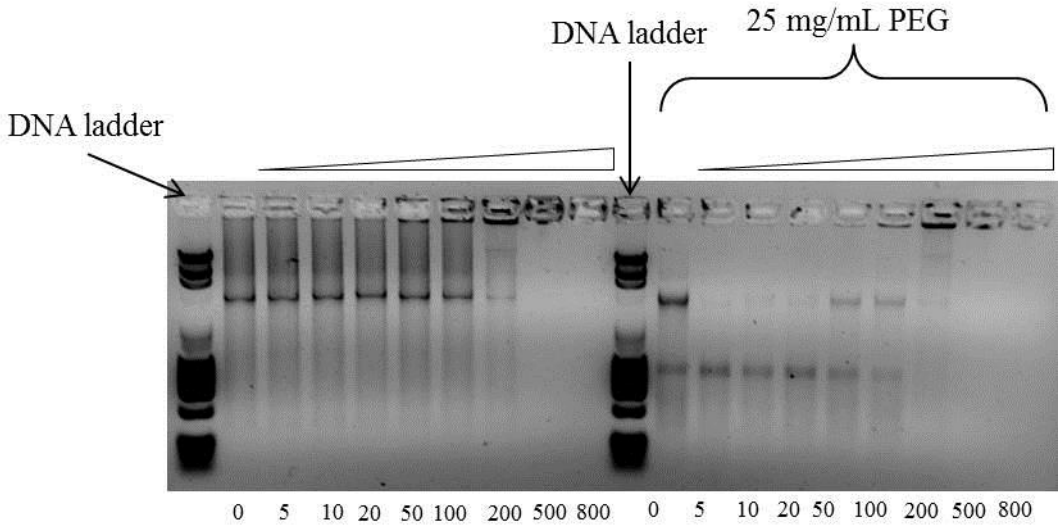

Figure 18: EMSA study, Gel Doc image of the gel after electrophoresis after 100 min, samples DNA-10 mM Tris-25 mg/mL PEG and increasing CTAB concentration, second band appear in the presence of PEG. The numbers below lanes represents CTAB concentration in μM.

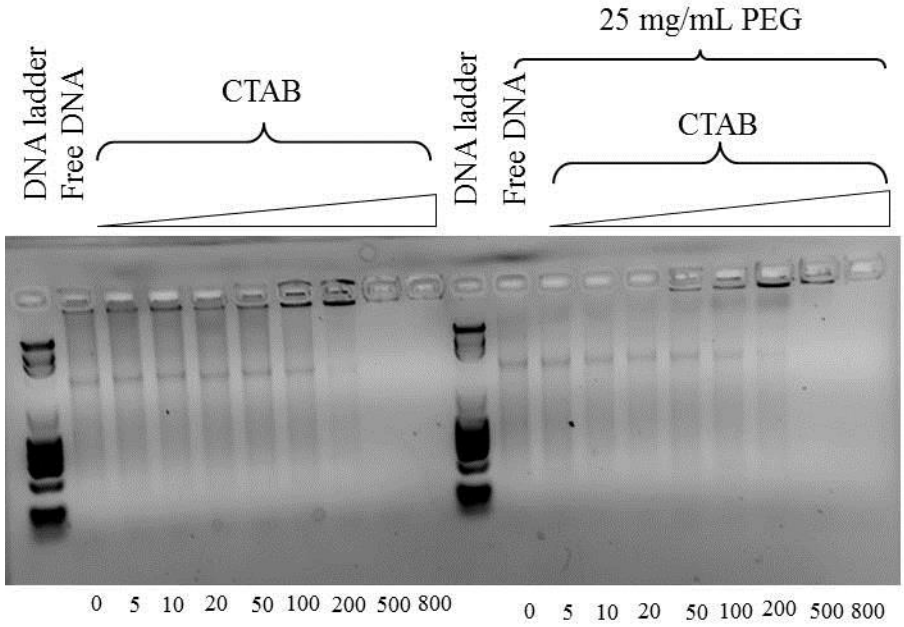

Figure 19: EMSA study, Gel Doc image of the gel after electrophoresis after 100 min, samples DNA-10 mM Tris-25 mg/mL PEG and increasing CTAB concentration, no second band appear in the presence of PEG.

4.5.2 EMSA of DNA, CTAB, PEG systems at high salt conditions

Electrophoreses of the DNA-CTAB and DNA-CTAB-PEG in high ionic strength were performed. The gel was checked after 40 min, 60 min and 100 min to see if the complexes in the presence of 100 mM NaBr were moving at the same speed.

It was found that with increasing CTAB concentration, the intensity of the band decreases. The decrease in the band intensity means lower DNA concentration. This is caused by the fact that complexes are formed and are either neutral so they cannot move out of the well to too big to move out of the well. The fluorescent dye can also be displaced from the DNA upon condensation by CTAB.

In case of DNA-CTAB systems, there is no significant decrease in the band intensity (0-100 μM CTAB), only the last visible band is with lower intensity (200 μM CTAB). Since the band corresponding to that of DNA alone is visible, there is some DNA which was not involved in the forming complexes and could move out of the well. Then, samples (500, 800 μM CTAB) form larger complexes which do not move out of the well at all meaning that there is no charged DNA which could move out of the well, so the system is completely neutral or is too big and every DNA molecule is inside.

In case of DNA-CTAB-PEG systems, it looks like the band intensity is decreasing linearly. So PEG probably support complex formation and does not allow DNA to leave the well. Since PEG is neutral polymer, DNA charge is not influence by PEG, so probably just the size of the complexes matters here. The trend is the same as for samples without crowding molecules. DNA molecules move out of the well until the CTAB concentration reaches 200 μM. Then, higher CTAB concentration (500, 800 μM), there is no movement out of the well at all.

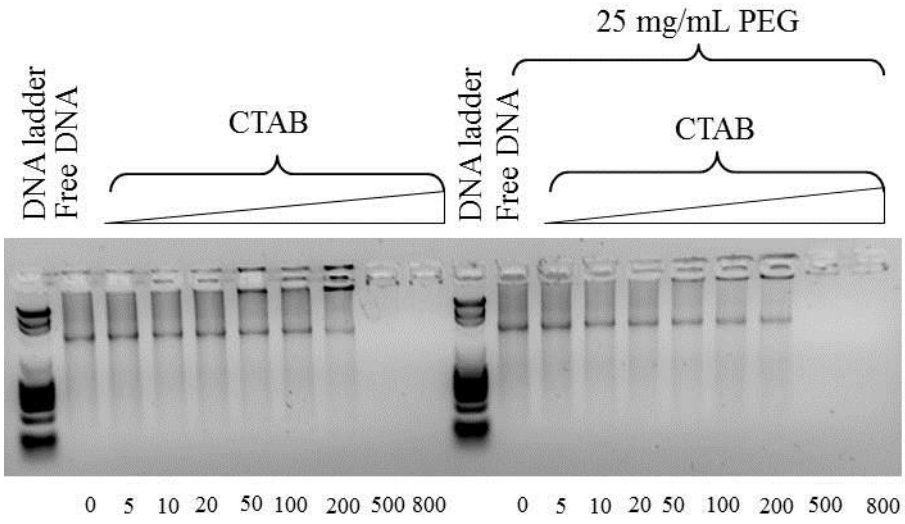

Figure 20: EMSA study, Gel Doc image of the gel after electrophoresis after 100 min, samples DNA-10 mM Tris-100 mM NaBr-25 mg/mL PEG and increasing CTAB concentration. The number below the lanes represents CTAB concentration in μM.

4.5.3 DNase protection assay

To gain better knowledge on the DNA condensation induced by CTAB in the absence and presence of PEG, DNase protection assays have been conducted. In this investigation, systems with and without crowding environment were compared. The image of the gel with samples with increasing CTAB concentration is shown in *Figure 21* and *Figure 22*.

There is no visible protection for samples in low salt concentration (*Figure 21*), neither without nor with PEG. The first lane presents the DNA ladder and the second well presents DNA without enzyme and the third is DNA with enzyme. This sample was used as a control and the enzyme totally digest the DNA. Fourth till eleventh well present samples with increasing CTAB concentration without crowding environment. It is obvious that in the first three CTAB samples (5 μM CTAB), the DNA totally digested by the enzyme. With increasing CTAB concentrations, the complexes start to form and stay in the well. The lane with DNA and PEG shows no protection against digestion. Samples with increasing concentration of CTAB in the presence of PEG show a little protection. Samples with lower CTAB concentration show band which correspond with very low DNA size, according to the DNA ladder. Samples with higher CTAB concentration form complexes which stay in the well.

Investigation in high salt condition is shown in *Figure 22*. First lane is DNA ladder, the second corresponds to DNA without enzyme and the third DNA with enzyme for comparison. In the wells with sample number four and five (200, 500 μM CTAB), the protection against digestion is visible since the DNA band is in the same lane as 4 000 bp band in DNA ladder which correlates with the DNA obtained from PCR. In comparison with just free DNA without enzyme and CTAB, the intensity of the band is lower which shows that some complexes are starting to form and stay in the wells but also some DNA (which is protected against enzyme cleavage) moves from the well and gives lighter band because of the lower concentration. Higher CTAB concentration (1 500, 2 000 and 3 500 μM) give rise to even bigger which are either uncharged, so they do not move, or too big to move. These complexes show no mobile DNA molecules which could move from the well. The second half of the gel shows samples in the crowding environment, constant PEG

concentration (25 mg/mL). At the first sight, there is obvious protection from the enzymatic cleavage by PEG in the samples with lower CTAB concentration. The last well presents sample with just DNA and 25 mg/mL PEG without and is mostly digested. The sample with the lowest CTAB concentration is protected from the digestion. Since the intensity of the band is really high and there is nothing visible in the well, it can be said that the whole sample moved from the well and was protected from the digestion. Increasing CTAB concentration causes complex formation and PEG causes protection there where it was totally digested in the samples without crowding environment. Second till fourth sample (10, 50, 200 μM CTAB) show the complex formation with protected DNA going out of the well. These DNA bands correspond with the 4 000 bp ladder band and control DNA band. Complexes which stayed in the wells are again either without charge or too big to move. Even higher CTAB concentration causes balanced charge so no movement of the complexes at all. This was seen in the complexes without PEG as well.

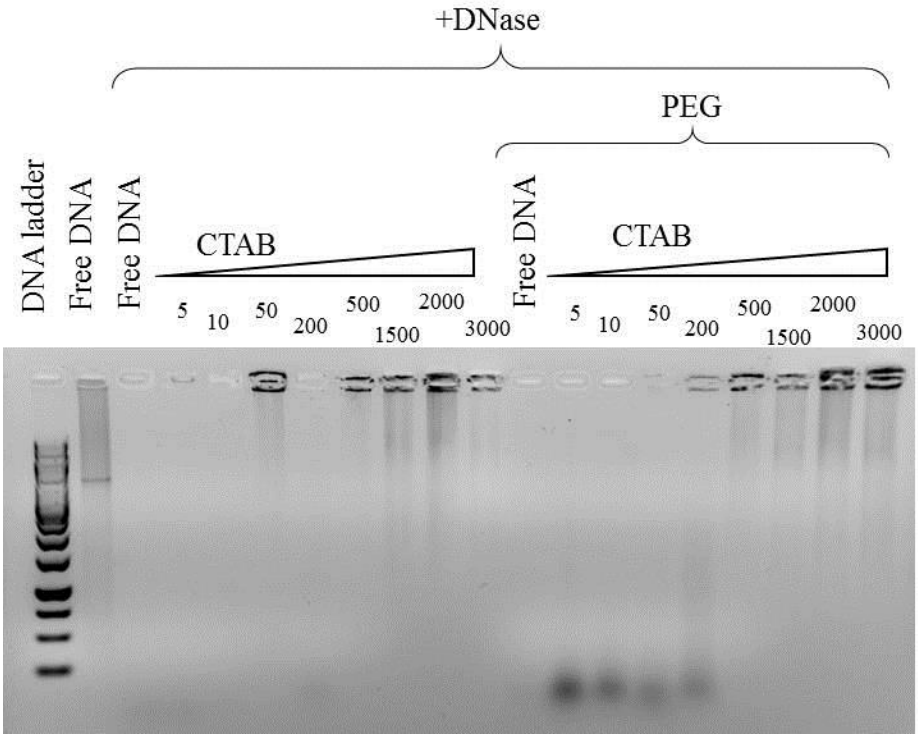

Figure 21: DNase protection assay of 4017 bp DNA under the influence of increasing CTAB concentration and 25 mg/mL PEG in the low salt condition (10 mM Tris). Sample sizes were 10 μL and DNA concentration 5 mg/ml. The number above the lanes represents CTAB concentration in μM.

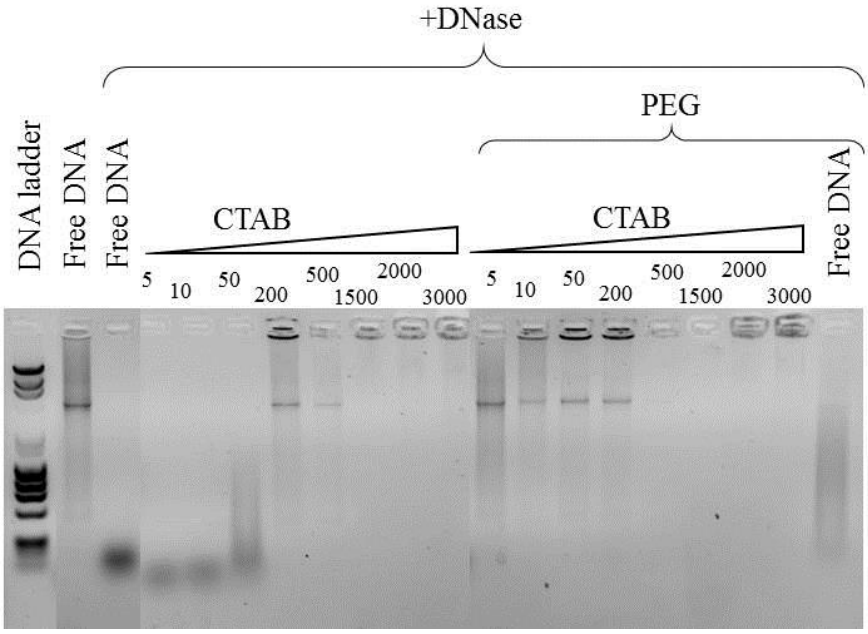

Figure 22: DNase protection essay of 4017 bp DNA under the influence of increasing CTAB concentration, 100 mM NaBr (high salt condition) and 25 mg/mL PEG. Sample sizes were 10 μL and DNA concentration 5 mg/ml. The number above the lanes represents CTAB concentration in μM. The gel image was rearranged in order to get clearer image.

4.6 CMC measurement

To find out if there is any change in CMC in the presence of PEG which could affect the interaction between DNA and CTAB, CMC measurement was performed by two different techniques.

4.6.1 Tensiometry

The surface tension measurement was performed as initial measurement with indicative CTAB concentrations and therefore, the data are not shown in the thesis.

4.6.2 HRUS

HRUS measurement was performed with higher accuracy to get more reliable results. The CMC value was calculated as intersection of the two linear regression equations from MS Excel.

Measurement of CTAB CMC was performed in water as well to confirm reliability of the method and the result is shown in *Figure 23*. The CMC value was calculated to be 0.94 mM, in good agreement with the literature [64, 65].

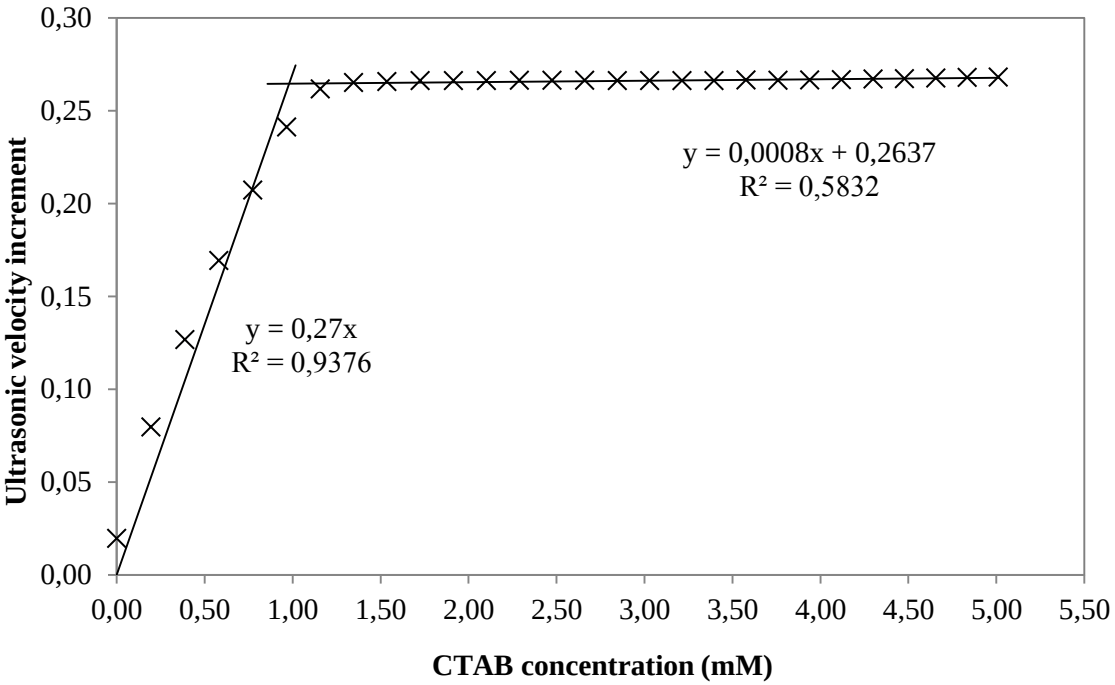

Figure 23: HRUS measurement of CTAB CMC in water at 25°C.

Next measurement was performed with 10 mM Tris buffer which was used in all measurements before and shown in *Figure 24*. CMC value was calculated as 0.80 mM which is slightly lower than in pure water. Geng and others studied the effect of Tris to CTAB CMC using surface tension measurement and found that the CMC decreased in the presence of Tris but concluded that buffer solution has no effect on surface tension [66].

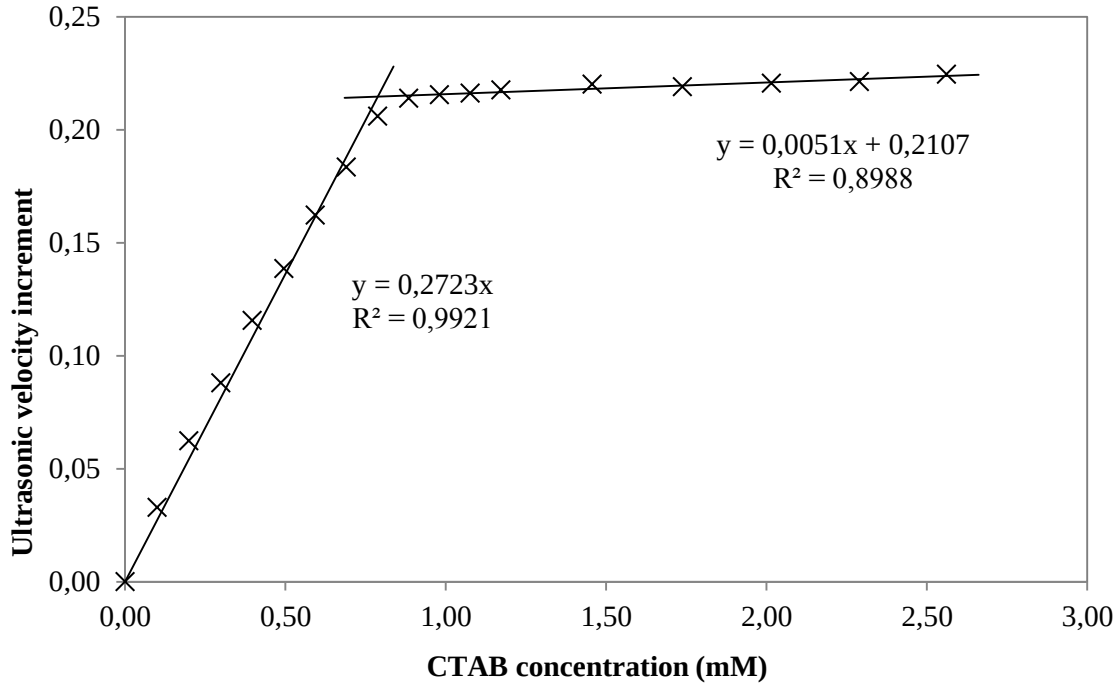

Figure 24: HRUS measurement of CTAB CMC at low salt conditions (10 mM Tris) at 25°C.

The effect of 25 mg/mL PEG 3000 on CMC was also studied and results are shown in *Figure 25*. CMC value was calculated as 0.77 mM. There is no significant change in CMC value in comparison with CMC at low salt condition in the absence of PEG. This is probably due to PEG neutrality.

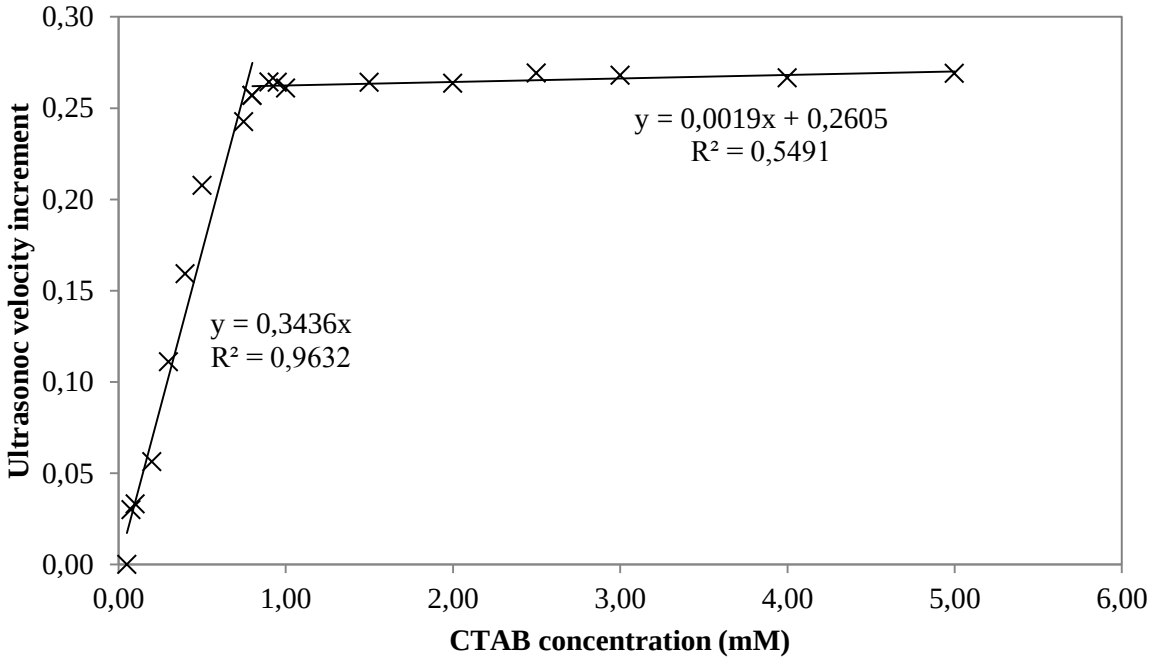

Figure 25: HRUS measurement of CTAB CMC in the presenc of 25 mg/mL PEG 3000 at low salt condition at 25°C.

Measurement was also performed in high salt conditions for comparison. It is generally known that addition of the salt decreases the CMC value ca 10×. Presence of PEG does not significantly influence the CMC value – 0.06 mM in absence of PEG (*Figure 26*) and 0.07 mM in the presence of PEG (*Figure 27*). These values are ca 10× lower than CMC at low salt conditions.

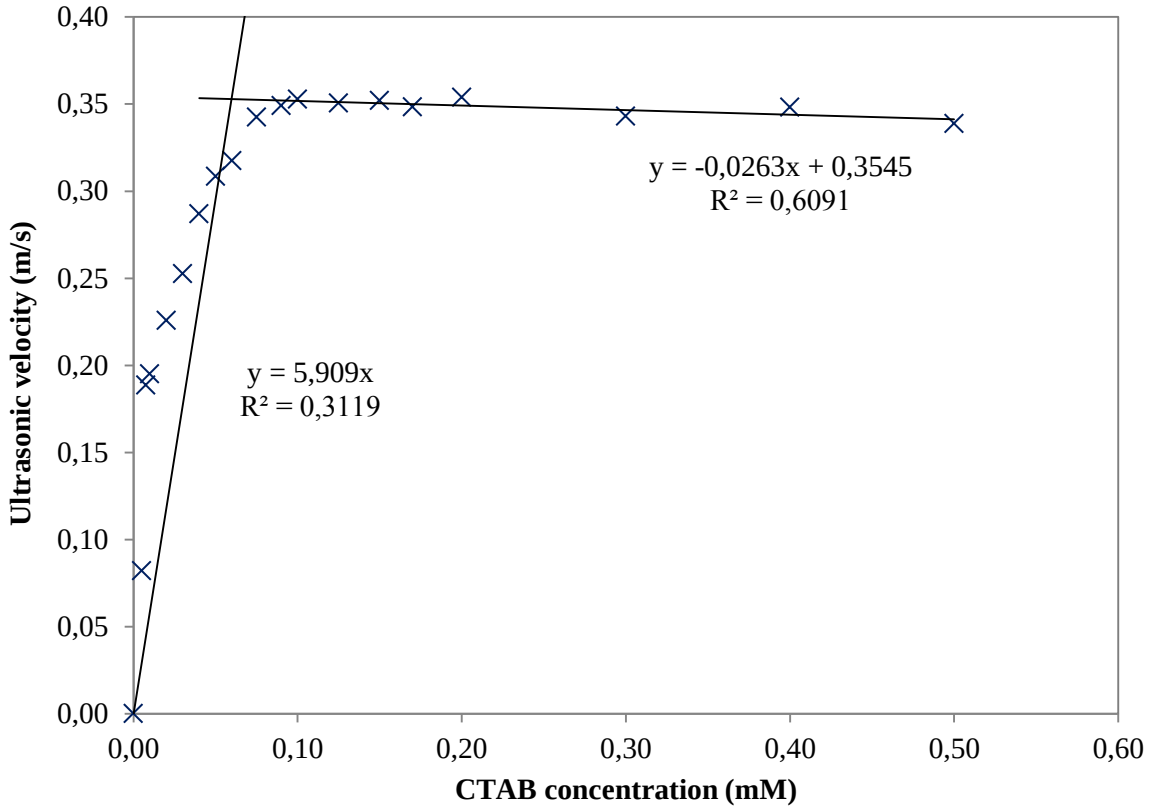

Figure 26: HRUS measurement of CMC at the high salt condition at 25°C.

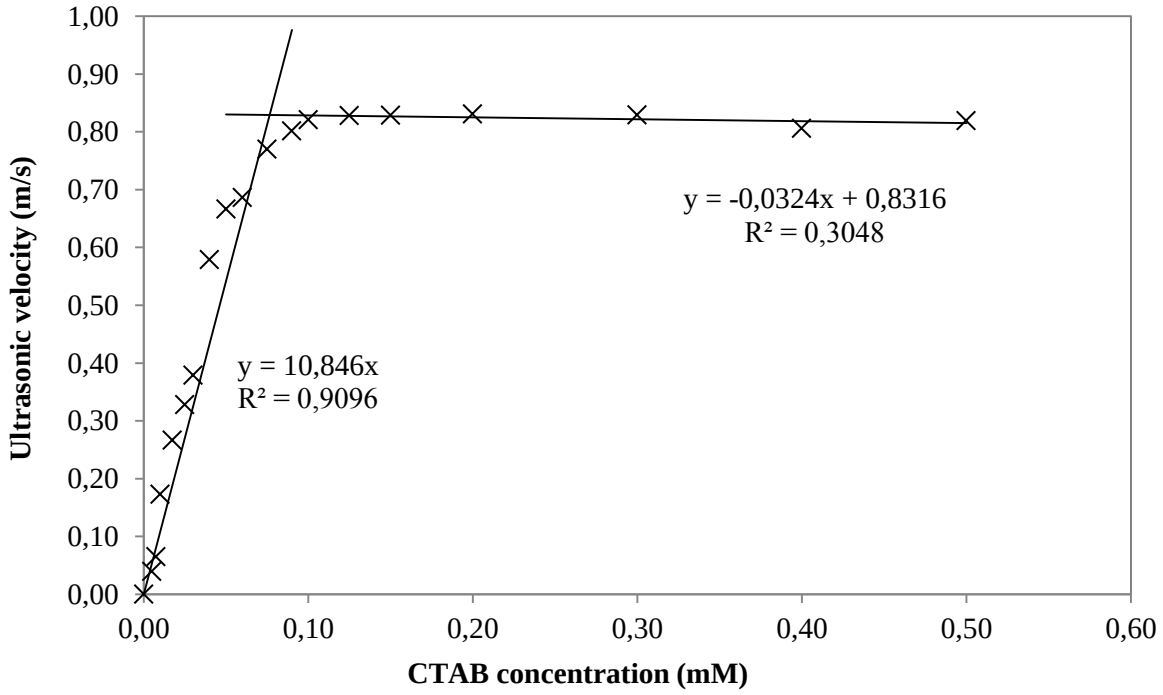

Figure 27: HRUS measurement of CTAB CMC at high salt condition in the presence of 25 mg/mL PEG 3000 at 25°C.

5 Conclusion

This work was inspired by gene delivery systems where DNA control of DNA compaction is necessary. Since both controlled DNA compaction and decompaction are needed for successful delivery [67], the aim of this work was to investigate the effect of crowding agents on DNA-surfactant interaction. Polyethylene glycol was used to mimic the crowding environment; CTAB was used as a surfactant and Tris was used as a buffer. After optimization of the working conditions, the model system was composed of DNA-CTAB-10 mM Tris, 25 mg/mL PEG (3000) was used as a crowding environment and 100 mM NaBr was used to check the influence of ionic strength. Firstly, PCR was used to copy DNA template for further use. Although it is not easy to investigate that pretty complex system and we were limited by the methods we can use, several methods were available and used – dye exclusion study, EMSA and DNase protection assay.

Dye exclusion study is based on the exclusion of fluorescent dye from the DNA when this becomes compacted. The fluorescence intensity is linearly dependent on the concentration of free DNA, and allowed to investigate the crowding effect and synergism of salt. It was confirmed that CTAB itself causes DNA compaction at certain concentration. At 20 μM CTAB, for example, there was found 40 % of DNA compaction. In the presence of crowding environment, on the other hand, DNA got compacted more, 75 % in the presence of 20 μM CTAB and 25 mg/mL PEG. In a further investigation, the synergism of the salt was considered and 100 mM NaBr was chosen as a salt. This gave a bit more interesting results. The significant synergism of PEG and salt was observed, this effect is also called polymer- and salt induced condensation. DNA got condense in the presence of really low concentration (7 μM CTAB) in comparison with the sample without crowding environment, where, on the other hand, the opposite effect that polymer- and salt induced condensation, which was also observed by [10, 11] in their studies.

By EMSA studies, the mobility of the samples in time was studied. The crowding environment and salt effect were compared. For the samples DNA-CTAB-10 mM Tris-25 mg/mL PEG, two different results were obtained, even though the experiment was repeated a couple times. The difference here is the appearance of the second band further in the gel, which means either more charged molecules or smaller ones, but it was not possible to make any conclusion out of this result. Samples which moved out of the well (0-200 μM CTAB) were moving the same speed and the place, where they stopped, corresponded with 4 000 bp ladder band and this corresponded with original DNA presented in the sample. It was found out that samples above a certain CTAB concentration (500, 800 μM), got stuck in the well due to either neutral charge of the formed complexes or due to a too big size. The intensity of the band was approximately the same along samples without crowding environment (0-100 μM CTAB), meaning that DNA concentration is approximately the same. Only sample with 200 μM CTAB showed lower intensity which means that some DNA remained in the well in arising complex. In the presence of PEG, the intensity of bands decreased with increasing CTAB concentration which means that some complexes were formed due to presence of PEG. In case of salt effect, there was no significant difference between DNA-CTAB-10 mM Tris-25 mg/mL PEG and DNA-CTAB-10 mM Tris-25 mg/mL PEG-100 mM NaBr investigation.

To summarize DNA protection assay, crowding molecules, PEG, prevented DNA cleavage in comparison with samples without PEG. This protection is only noticeable in the samples with lower CTAB concentration (5–200 μM) where the free DNA moved from the well and

stopped there where the band of 4 000 bp is. Complexes also gave arise in those samples which decreases the DNA band intensity (lower DNA concentration) because some of the DNA stayed in the well in the formed complexes. In both cases, complexes which stayed in the wells were either neutral or too big to move.

HRUS was used as a main method to determine whether surfactant CMC changes in the present of Tris and PEG. It can be concluded that CMC is not affected by addition of neither Tris nor PEG, the values are just slightly lower than CMC for CTAB in pure water.

It can be claimed that the aim of the work was fulfilled. The work provides basic introduction to the problem and would be suitable to follow this work and look at different crowding molecules, such as negatively charged silicon nanoparticles or PEG with different molecular weight. It would be also possible to use different surfactants than CTAB or investigate the effect of alkyl chain length of the surfactant on that interaction. It would be also interesting to mimic the physiological conditions by 0.15 M NaCl or focus on controlling compaction and decompaction.

This work will be part of the publication in scientific journal with the title Combined role of macromolecular crowding and cationic surfactant in efficient DNA condensation.

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7 LIST OF ABBREVIATION AND SYMBOLS

7.1 List of abbreviations

PEG polyethylene glycol
PAA Polyacrylic acid
DNA deoxyribonucleic acid
CTAB cetyltrimethylammonium bromide
PCR polymerase chain reaction
bp base pair
dNTP deoxynucleotide
TAE Tris-acetate-EDTA buffer
EMSA Electrophoretic mobility shift assay
VR vibrational relaxation
$S_{0, 1, 2}$ singlet states
HRUS high resolution ultrasonic spectroscopy
RCF Revolutions per minute

7.2 List of symbols

M_w molecular weight
nm nanometre
ns nanosecond
τ lifetime
ϕ quantum yield
μM micromolar, 10^{-6} mol/L
mM millimolar10^{-3} mol/L
μL microliter
γ surface tension

8 APPENDIX

8.1 Sample preparation tables

Table 1: Dye exclusion assay: sample preparation for PEG research.

PEG final c [mg/mL]	PEG stock c [mg/mL]	100X Gel Star™ [µL]	20 mg/mL DNA [µL]		10 mM Tris [µL]	PEG [µL]	
0	0	5	5	15 min incubation	40	0	30 min incubation
1	10	5	5		35	5	
5	50	5	5		35	5	
10	100	5	5		35	5	
15	150	5	5		35	5	
25	250	5	5		35	5	
35	350	5	5		35	5	
40	400	5	5		35	5	
50	400	5	5		33,75	6,25	
70	400	5	5		31,25	8,75	

Table 2: Dye exclusion assay: sample preparation for CTAB research.

CTAB final c [µM]	CTAB stock c [µM]	100X Gel Star™ [µL]	20 mg/mL DNA [µL]		10 mM Tris [µL]	CTAB [µL]	
0	0	5	5	15 min incubation	40	0	60 min incubation
0,5	5	5	5		35	5	
0,7	7	5	5		35	5	
0,9	9	5	5		35	5	
1	10	5	5		35	5	
1,5	15	5	5		35	5	
2	20	5	5		35	5	
3	30	5	5		35	5	
3,5	35	5	5		35	5	
4	40	5	5		35	5	

CTAB final c [µM]	CTAB stock c [µM]	100X Gel Star™ [µL]	20 mg/mL DNA [µL]		10 mM Tris [µL]	CTAB [µL]	
5	50	5	5		35	5	
7	70	5	5		35	5	
10	100	5	5		35	5	
15	150	5	5		35	5	
20	200	5	5		35	5	
30	300	5	5		35	5	
40	400	5	5		35	5	
50	500	5	5		35	5	
70	700	5	5		35	5	
100	1 000	5	5		35	5	

Table 3: Dye exclusion assay: sample preparation for salt effect investigation as a function of PEG concentration.

PEG final c [mg/mL]	PEG stock c [mg/mL]	100X Gel Star™ [µL]	20 mg/mL DNA [µL]		NaBr [µL]	200 mM Tris [µL]	PEG [µL]	
0	0	5	5	15 min incubation	5	35	0	30 min incubation
1	10	5	5		5	30	5	
5	50	5	5		5	30	5	
10	100	5	5		5	30	5	
15	150	5	5		5	30	5	
25	250	5	5		5	30	5	
35	350	5	5		5	30	5	
40	400	5	5		5	30	5	
50	400	5	5		5	28,75	6,25	
70	400	5	5		5	26,25	8,75	

Table 4: Dye exclusion assay: control sample preparation.

CTAB final c [μM]	CTAB stock c [μM]	100X Gel Star™ [μL]	20 mg/mL DNA [μL]		10 mM Tris [μL]	CTAB [μL]	
0	0	5	5	15 min incubation	40	0	60 min incubation
5	50	5	5		35	5	
7	70	5	5		35	5	
9	90	5	5		35	5	
10	100	5	5		35	5	
15	150	5	5		35	5	
20	200	5	5		35	5	
30	300	5	5		35	5	
40	400	5	5		35	5	
50	500	5	5		35	5	
70	700	5	5		35	5	
100	1 000	5	5		35	5	

Table 5: Dye exclusion assay: sample preparation in crowding environment.

CTAB final c [μM]	CTAB stock c [μM]	100X Gel Star™ [μL]	20 mg/mL DNA [μL]		10 mM Tris [μL]	CTAB [μL]		250 mg/mL PEG [μL]	
0	0	5	5	15 min incubation	35	0	60 min incubation	5	30 min incubation
5	50	5	5		30	5		5	
7	70	5	5		30	5		5	
9	90	5	5		30	5		5	
10	100	5	5		30	5		5	
15	150	5	5		30	5		5	
20	200	5	5		30	5		5	
30	300	5	5		30	5		5	
40	400	5	5		30	5		5	

CTAB final c [µM]	CTAB stock c [µM]	100X Gel Star™ [µL]	20 mg/mL DNA [µL]		10 mM Tris [µL]	CTAB [µL]		250 mg/mL PEG [µL]	
50	500	5	5		30	5		5	
70	700	5	5		30	5		5	
100	1 000	5	5		30	5		5	

Table 6: Dye exclusion assay: control sample preparation for salt effect and comparison.

CTAB final c [µM]	CTAB stock c [µM]	100X Gel Star™ [µL]	20 mg/mL DNA [µL]		10 mM Tris [µL]	CTAB [µL]	1000 mM NaBr [µL]	
0	0	5	5	15 min incubation	35	0	5	60 min incubation
5	50	5	5		30	5	5	
7	70	5	5		30	5	5	
9	90	5	5		30	5	5	
10	100	5	5		30	5	5	
15	150	5	5		30	5	5	
20	200	5	5		30	5	5	
30	300	5	5		30	5	5	
40	400	5	5		30	5	5	
50	500	5	5		30	5	5	
70	700	5	5		30	5	5	
100	1 000	5	5		30	5	5	

Table 7: Dye exclusion assay: sample preparation for salt effect in crowding environment.

CTAB final c [μM]	CTAB stock c [μM]	100X Gel Star™ [μL]	20 mg/mL DNA [μL]		10 mM Tris [μL]	CTAB [μL]	1000 mM NaBr [μL]		250 mg/mL PEG [μL]	
0	0	5	5	15 min incubation	30	0	5	60 min incubation	5	30 min incubation
5	50	5	5		25	5	5		5	
7	70	5	5		25	5	5		5	
9	90	5	5		25	5	5		5	
10	100	5	5		25	5	5		5	
15	150	5	5		25	5	5		5	
20	200	5	5		25	5	5		5	
30	300	5	5		25	5	5		5	
40	400	5	5		25	5	5		5	
50	500	5	5		25	5	5		5	
70	700	5	5		25	5	5		5	
100	1 000	5	5		25	5	5		5	

Table 8: EMSA studies: control sample praparation.

CTAB final c [µM]	CTAB stock c [µM]	33 mg/mL DNA [µL]	10 mM Tris [µL]	CTAB [µL]	
0	0	3	17	0	60 min incubation
5	50	3	15	2	
10	100	3	15	2	
20	200	3	15	2	
50	500	3	15	2	
100	1 000	3	15	2	
200	2 000	3	15	2	
500	5 000	3	15	2	
800	8 000	3	15	2	

Table 9: EMSA studies: control sample preparation with salt conditions.

CTAB final c [µM]	CTAB stock c [µM]	33 mg/mL DNA [µL]	10 mM Tris [µL]	CTAB [µL]	1000 mM NaBr [µL]	
0	0	3	15	0	2	60 min incubation
5	50	3	13	2	2	
10	100	3	13	2	2	
20	200	3	13	2	2	
50	500	3	13	2	2	
100	1 000	3	13	2	2	
200	2 000	3	13	2	2	
500	5 000	3	13	2	2	
800	8 000	3	13	2	2	

Table 10: EMSA studies: sample preparation in crowding environment.

CTAB final c [μM]	CTAB stock c [μM]	33 mg/mL DNA [μL]	10 mM Tris [μL]	CTAB [μL]		250 mg/mL PEG [μL]	
0	0	3	15	0	60 min inctubation	2	30 min incubation
5	50	3	13	2		2	
10	100	3	13	2		2	
20	200	3	13	2		2	
50	500	3	13	2		2	
100	1 000	3	13	2		2	
200	2 000	3	13	2		2	
500	5 000	3	13	2		2	
800	8 000	3	13	2		2	

Table 11: EMSA studies: sample preparation for salt effect comparison in crowding environment.

CTAB final c [μM]	CTAB stock c [μM]	33 mg/mL DNA [μL]	10 mM Tris [μL]	CTAB [μL]	1000 mM NaBr [μL]		250 mg/mL PEG [μL]	
0	0	3	13	0	2	60 min incubation	2	30 min incubation
5	50	3	11	2	2		2	
10	100	3	11	2	2		2	
20	200	3	11	2	2		2	
50	500	3	11	2	2		2	
100	1 000	3	11	2	2		2	
200	2 000	3	11	2	2		2	
500	5 000	3	11	2	2		2	
800	8 000	3	11	2	2		2	

Table 12: DNase protection assay: control sample preparation for salt conditions.

CTAB final c [µM]	CTAB stock c [µM]	50 mg/mL DNA [µL]	10 mM Tris [µL]	CTAB [µL]	1000 mM NaBr [µL]		DNase [µL]	
0	0	5	39	0	5	60 min inctubation	1	20 min incubation, 37 °C
5	50	5	34	5	5		1	
10	100	5	34	5	5		1	
50	500	5	34	5	5		1	
200	200	5	34	5	5		1	
500	5 000	5	34	5	5		1	
1 500	15 000	5	34	5	5		1	
2 000	20 000	5	34	5	5		1	
3 000	30 000	5	34	5	5		1	

Table 13: DNase protection assay: sample preparation for salt effect in crowding conditions.

CTAB final c [µM]	CTAB stock c [µM]	50 mg/mL DNA [µL]	10 mM Tris [µL]	CTAB [µL]	1000 mM NaBr [µL]		250 mg/mL PEG [µL]		DNase [µL]	
0	0	5	34	0	5	60 min incubation	5	30 min inctubation	1	20 min incubation, 37 °C
5	50	5	29	5	5		5		1	
10	100	5	29	5	5		5		1	
50	500	5	29	5	5		5		1	
200	200	5	29	5	5		5		1	
500	5 000	5	29	5	5		5		1	
1 500	15 000	5	29	5	5		5		1	
2 000	20 000	5	29	5	5		5		1	
3 000	30 000	5	29	5	5		5		1	